N° d'ordre : 231-2015
Année : 2015

THÈSE DE L'UNIVERSITÉ DE LYON
Délivrée par
L'UNIVERSITÉ CLAUDE BERNARD LYON 1
ÉCOLE DOCTORALE DE CHIMIE
Discipline : Pharmacotechnie

ET

THÈSE DE L'UNIVERSITÉ DE MONASTIR
Délivrée par
LA FACULTÉ DE PHARMACIE DE MONASTIR
ÉCOLE DOCTORALE
Discipline : Sciences pharmaceutiques

DIPLOME DE DOCTORAT

(arrêté du 7 août 2006)

soutenue publiquement le 27 Novembre 2015

par:

Mr. Karim MILADI

Élaboration de nanoparticules contenant l'alendronate de sodium pour une application en ostéoporose

JURY:

Dr. Gillian BARRATT, CNRS, Université Paris Sud, France, rapporteur
Pr. Sami BOUFI, Faculté des sciences de Sfax, Université de Sfax, Tunisie, rapporteur
Pr. Abderrahman BOURAOUI, Faculté de pharmacie de Monastir, Université de Monastir, Tunisie, examinateur
Dr. Abdelhamid ELAISSARI, CNRS, Université Claude Bernard-Lyon1, France, directeur de thèse
Pr. Hatem FESSI, CNRS, Université Claude Bernard-Lyon 1, France, co-directeur
Pr. Souad SFAR, Faculté de pharmacie de Monastir, Université de Monastir, Tunisie, co-directeur

UNIVERSITE CLAUDE BERNARD - LYON 1

Président de l'Université	**M. François-Noël GILLY**
Vice-président du Conseil d'Administration	M. le Professeur Hamda BEN HADID
Vice-président du Conseil des Etudes et de la Vie Universitaire	M. le Professeur Philippe LALLE
Vice-président du Conseil Scientifique	M. le Professeur Germain GILLET
Directeur Général des Services	M. Alain HELLEU

COMPOSANTES SANTE

Faculté de Médecine Lyon Est – Claude Bernard	Directeur : M. le Professeur J. ETIENNE
Faculté de Médecine et de Maïeutique Lyon Sud – Charles Mérieux	Directeur : Mme la Professeure C. BURILLON
Faculté d'Odontologie	Directeur : M. le Professeur D. BOURGEOIS
Institut des Sciences Pharmaceutiques et Biologiques	Directeur : Mme la Professeure C. VINCIGUERRA
Institut des Sciences et Techniques de la Réadaptation	Directeur : M. le Professeur Y. MATILLON
Département de formation et Centre de Recherche en Biologie Humaine	Directeur : Mme. la Professeure A-M. SCHOTT

COMPOSANTES ET DEPARTEMENTS DE SCIENCES ET TECHNOLOGIE

Faculté des Sciences et Technologies	Directeur : M. F. DE MARCHI
Département Biologie	Directeur : M. le Professeur F. FLEURY
Département Chimie Biochimie	Directeur : Mme Caroline FELIX
Département GEP	Directeur : M. Hassan HAMMOURI
Département Informatique	Directeur : M. le Professeur S. AKKOUCHE
Département Mathématiques	Directeur : M. le Professeur Georges TOMANOV
Département Mécanique	Directeur : M. le Professeur H. BEN HADID
Département Physique	Directeur : M. Jean-Claude PLENET
UFR Sciences et Techniques des Activités Physiques et Sportives	Directeur : M. Y.VANPOULLE
Observatoire des Sciences de l'Univers de Lyon	Directeur : M. B. GUIDERDONI
Polytech Lyon	Directeur : M. P. FOURNIER
Ecole Supérieure de Chimie Physique Electronique	Directeur : M. G. PIGNAULT
Institut Universitaire de Technologie de Lyon 1	Directeur : M. le Professeur C. VITON
Ecole Supérieure du Professorat et de l'Education	Directeur : M. le Professeur A. MOUGNIOTTE
Institut de Science Financière et d'Assurances	Directeur : M. N. LEBOISNE

FACULTE DE PHARMACIE DE MONASTIR

Doyen : Pr Abdelhalim TRABELSI

PHARMACIENS HOSPITALO-UNIVERSITAIRES

PROFESSEURS EMERITES

MICROBIOLOGIE…….. Moncef JEDDI
HEMATOLOGIE…… Kamel BOUKEF
BIOLOGIE CELLULAIRE…… Hassen CHAABANI
BIOCHIMIE…… Abdelhédi MILED

P R O F E S S E U R S

MICROBIOLOGIE…….. Chédlia FENDRI
Noureddine BOUJAAFAR
Noura Leïla SLIM
Mohamed KHEDER
Ridha BEN AISSA
Abdelhalim TRABELSI
Ridha M'ZOUGHI
Férièle MESSAADI
Leïla BACCAR ép. BAKIR
Farouk BARGUELLIL
Afef MASMOUDI
Sophia BOUHALILA-BESBES
Maha MASTOURI
Amel EL FRAY-RHIM
Soumaya KETATA
Mohamed BEN MOUSSA
Youssef BEN SALEM
Imène FODHA
Meriem ZRIBI
Emna M'HIRI

IMMUNOLOGIE …….. Faouzi JENHANI
Ibtissem GHEDIRA
Salma FEKI
Nabil SAKLY
Ezzeddine GHAZOUANI

HEMATOLOGIE…….. Nour El Houda TOUMI
Mondher KORTAS
Slama HMIDA
Saloua JEMNI
Touhami MAHJOUB
Abderraouf OUESLATI
Nasreddine GRITLI
Mohsen HASSINE
Najet MOJAAT
Brahim NCIRI

	Salem ABID
	Mohamed ZILI Choumous KALLEL Houda KAABI Néjia JMILI-BRAHAM Batoul HOUISSA Sondos LAMLOUM ép. HIZEM Fatma ABDERRAZAK Tahar CHAKROUN
BIOCHIMIE……	Mohamed Fadhel NAJJAR Néziha KAABACHI Ali BOUSLAMA Mohamed Chiheb BEN RAYANA Mohamed Adel BEN AMOR Taïeb MESSAOUD Kacem MAHDOUANI Haïfa SENHAJI Asma OMEZZINE Selima FERCHICHI Amina BIBI Zied AOUNI Fadoua NEFFATI - HASSINE
PARASITOLOGIE……..	Hamouda BABBA Sâadia FOURATI-GARGOURI Boutheïna JEMLI
TOXICOLOGIE……..	Abderrazak LEHDHILI Youssef BRAHAM Wahiba DOUKI Hayet GHORBAL-BRAHAM Dorra AMIRA
PHARMACIE GALENIQUE……	Souad SFAR GANDOURA
PHARMACOLOGIE……..	Nabil CHOUCHENE Nadia FENINA
CHIMIE ANALYTIQUE…….	Mohamed KALLEL Féthi SAFTA Farhat FARHAT
PHARMACOGNOSIE……	Rachid CHEMLI Kamel GHEDIRA
PHYSIOLOGIE HUMAINE ET EXPLORATION FONCTIONNELLE……..	Thabet TABKA

MAITRES DE CONFERENCES AGREGES

BIOCHIMIE……	Chakib MAZIGH Nabila BEN REJEB Hassen BOUZIDI Henda CHAHED Ilham HELLARA
HEMATOLOGIE……	Inès JDIDI Manel CHAABANE

MICROBIOLOGIE…….. Anis BEL HAJ KHELIFA
Asma GHARIANI
Mohamed Salim ASLI
Chérifa CHAOUCH

IMMUNOLOGIE…….. Radhia KOCHKAR
Yousr GALAI

TOXICOLOGIE…… Kaïs GARRAB

CYTOGENETIQUE …..…… Ghaya MERDESSI

PHARMACIE GALENIQUE…….. Imène LIMAYEM
Emna ZRIBI
Senda BAHRI
Mohamed Ali EL YOUSFI

PHARMACIE CLINIQUE ……..…… Zeïneb OUAHCHI

CHIMIE ANALYTIQUE ……..…… Inès EL FRAD
Amel HASSAIRI

BIOPHYSIQUE PHARMACEUTIQUE ……..…… Najla AYACHI

CHIMIE THERAPEUTIQUE…… Olfa GLOULOU
Fayçal JENAN

PHARMACOLOGIE…… Mehdi DRIDI

ASSISTANTS

BIOCHIMIE…….. Yassine CHAABOUNI
Lobna BOUACIDA
Soumaya BERRIRI
Rim DEBBOUBI ép. ALOUI
Faten AMDOUNI

HEMATOLOGIE…… Saida BEN HATIRA
Mourad CHAARI
Amina BOUATTAY ép. BOUZOUITA
Meriem BECHEUR
Mouna SASSI
Houda ROMDHANE
Nada DAMMAK
Inès GHARIANI
Sami LAYOUNI
Asma AYED
Sonia CHOUAIEB
Saoussen CHOUCHENE ép. HADDAD

MICROBIOLOGIE…… Selma SMAOUI
Aïda ELARGOUBI
Yomna BEN LAMINE
Adam BEN NASR
Hager BATIKH
Sana KAMMOUN
JMAL

	Chema MAROUANE MAAZOUN Ons HADDAD
VIROLOGIE……	Saoussen KACEM
TOXICOLOGIE……	Meriem ZOUARI Asma GHORBEL Mohamed Hachem SAADAOUI
CYTOGENETIQUE ……	Elyes Slim GHEDIRA
PHARMACIE GALENIQUE……	Ahmed AMRI Balsam KACEM Nesrine KALBOUSSI ép. HAFSIA
PHARMACIE CLINIQUE ……	Zakia Inès HARZALLAH Khouloud BEN JEDDOU Nesrine HASNI Dorra JARRAYA Ghassen BELHABIB
CHIMIE ANALYTIQUE ……	Hanène OUESLATI Inès GANA Azza BEN SAID
PHARMACOGNOSIE……	Houda BOUATTOUR Safa DERBAL Amine TRABELSI
PHYSIOLOGIE……	Rabii SELMI Amani CHERIF
CHIMIE THERAPEUTIQUE……	Mohamed Ali ESSOUSSI
PHARMACOLOGIE……	Manel TURKI Meriem RAZGALLAH – KHROUF Wafa CHAOUCH (Mise en disponibilité à partir du 1/6/2013) Mohamed Amine EL KAIBI
CHIMIE ORGANIQUE……	Donia GHEDIRA
IMMUNOLOGIE……	Meriem GHOZZI

PERSONNEL PHARMACIEN UNIVERSITAIRE

P R O F E S S E U R S

PHARMACOLOGIE……	Abderrahman BOURAOUI Letaïef REKIK
BIOPHYSIQUE……	Ridha HAMDANE

A S S I S T A N T

PHARMACIE GALENIQUE ..... Mohamed Ali LASSOUED

PERSONNEL ENSEIGNANT SCIENTIFIQUE

P R O F E S S E U R S

BIOCHIMIE..... Jemni BEN CHIBANI
Khira MAAROUFI

MICROBIOLOGIE..... Mahjoub AOUNI
Amina BEN FEDHILA-BAKHROUF

PHYSIOLOGIE ANIMALE..... Dalila SAIDANE-MOSBAHI

PHYSIOLOGIE ..... Ahmed Noureddine HELLAL

Maître de Conférences

PHYSIOLOGIE ..... Hassen BEN ABDENNABI

BOTANIQUE, PHYSIOLOGIE VEGETALE..... Zohra MARZOUK

CHIMIE..... Chedly BOUDOUKHANE

MAITRES ASSISTANTS

BOTANIQUE, PHYSIOLOGIE VEGETALE..... Saïda CHAKROUN

BOTANIQUE ..... Dhahbia KHIARI

PHARMACIE GALENIQUE..... Taoufik DOUSS

CHIMIE ..... Béchir M'RABET
Jamil KRAIEM
Najah CHERIAA ép. JEGUIRIM
Faouzi AYARI
Hager TRAD

CHIMIE ANALYTIQUE ..... Mouna TOUATI ép. BELHAJ YOUSSEF

PHARMACOGNOSIE..... Fatma BANNOUR

SCIENCES BIOLOGIQUES PHARMACEUTIQUES..... Meriem BEN HAMIDA REBII

PARASITOLOGIE..... Najoua HOUAS
(Coopération 5 ans à partir de 2013)
Slim MRAD

BIOLOGIE CELLULAIRE ..... Soumaya KILANI

GENETIQUE..... Sana SFAR

BIOPHYSIQUES..... Sonia SFAR LAATAOUI

Raja GUEDOUAR

IMMUNOLOGIE…… Wahiba SAKLY

PHYSIQUE…… Hanène OUERFELLI

ASSISTANT

BIOLOGIE CELLULAIRE …… Latifa CHKIOUA

BOTANIQUE …… Ameur EL AISSI
Hanène ALMADINI

SCIENCES BIOLOGIQUES PHARMACEUTIQUES…… Ibtissem LAHMAR

MATHEMATIQUES APPLIQUEES…… Mohamed KHARRAT

PROFESSEUR DE L'ENSEIGNEMENT SECONDAIRE

ANGLAIS…… Mounira ZOUITEN-BESBES
Fredj ALAYA

INFORMATIQUE …… Anis NASRI
Amel JABLAOUI

EDUCATION PHYSIQUE…… Hichem EL BAKKARI

ENSEIGNANT CONTRACTUEL TUNISIEN

STATISTIQUES ……Sahbi FARHANI

CHIMIE …… Hassen CHALLOUF
Henda KESKES
Anouar BEN FREDJ
Oussama JILANI
Nesrine DAMMAK
Fatma NJAH

Résumé

L'ostéoporose est la maladie métabolique la plus fréquente qui touche l'os. Plusieurs substances actives sont utilisées pour le traitement pharmacologique de cette maladie. Cependant, ce sont les bisphosphonates et surtout l'alendronate de sodium, qui sont prescrits en première intention. L'alendronate de sodium est, en effet, très efficace mais présente une faible absorption quand il est administré par la voie orale. Sa solubilité dans l'eau est de 20 mg/ml. Il présente en outre une faible biodisponibilité (de 0,6 à 0,7%). Cette substance active est aussi à l'origine d'effets indésirables d'irritation au niveau de l'œsophage, l'estomac et l'intestin. Ces effets sont dus à un contact local des cristaux de la substance active avec la muqueuse. L'approche d'encapsulation des substances actives dans des particules polymériques a permis d'obtenir plusieurs bénéfices thérapeutiques comme l'amélioration de la biodisponibilité et la diminution des effets indésirables. Dans la première partie de notre étude, on a réalisé l'encapsulation de l'alendronate dans des nanoparticules à base de poly-ε-caprolactone en utilisant la nanoprécipitation et l'émulsion double. Les nanoparticules obtenues ont une forme sphérique et une taille comprise entre 200 et 450 nm. Le meilleur pourcentage d'encapsulation a été de 34% et il a été obtenu avec la technique d'émulsion double. Ceci confirme que cette méthode est plus adaptée à l'encapsulation des molécules hydrophiles. Le profil de libération *in vitro* a montré deux phases : une première phase de libération relativement rapide et une deuxième phase beaucoup plus lente. L'analyse par modélisation mathématique a montré que la libération *in vitro* de l'alendronate se fait par diffusion et relâchement des chaines polymériques. Dans la deuxième partie expérimentale, une alternative plus intéressante que l'encapsulation dans de la poly-ε-caprolactone a été proposée. Il s'agit de l'utilisation du chitosane qui est un polymère naturel hydrophile. Ceci a permis d'éviter l'utilisation de solvants organiques. En plus, une optimisation du pourcentage d'encapsulation a été obtenue (70%) en utilisant la gélification ionique. C'est une technique d'encapsulation simple qui est basée sur le passage d'un polymère en solution à l'état gel suite à une interaction électrostatique avec un polyanion. Les nanoparticules obtenues ont une forme sphérique et une taille allant de 91 à 175 nm en fonction des paramètres opératoires. Le profil général de libération *in vitro* a été similaire à celui obtenu avec la première étude mais l'avantage est que la libération a été plus rapide. Ceci rend possible une application *in vivo* des particules préparées. Ces particules peuvent présenter une alternative intéressante pour le traitement de l'ostéoporose par voie orale. Des études *in vivo* chez le rat ont été réalisées pour étudier la biodisponibilité et la tolérance gastro-intestinale de ces particules.

Abstract

Osteoporosis is the most frequent metabolic disease that affects bone. Many actives have been used as pharmacological treatment of this disease. However, bisphosphonates, especially, alendronate sodium, are indicated as first line regimen. Alendronate is highly efficient but presents low absorption after oral administration. Its solubility in water is 20 mg/ml. It has also poor bioavailability (0.6-0.7%). In addition, this active could lead to many side effects, which are mainly related to the esophagus, the stomach and the intestine. Such effects are linked to a local contact of drug crystals with the mucosa. Encapsulation of active molecules allowed the obtaining of many advantages over conventional pharmaceutical forms such as, bioavailability and tolerance enhancement. In the first part of our study, we managed to encapsulate alendronate sodium in poly-ε-caprolactone nanoparticles via two techniques: nanoprecipitation and double emulsion. Obtained nanoparticles presented a spherical form. Their size ranged between 200 and 450 nm. The highest encapsulation efficiency value was 34% and was obtained via double emulsion technique. This confirms that double emulsion is more suitable for hydrophilic drugs encapsulation. *In vitro* release profile showed two phases: first phase of burst release and a second more prolonged phase. Mathematical modeling showed that alendronate *in vitro* release occurs by drug diffusion and polymer chain relaxation. In the second experimental part, we managed to find a more interesting alternative. In fact, we opted for the use of chitosan which is a natural hydrophilic polymer. One of the obtained advantages is the avoidance of organic solvents use. In addition, this approach allowed the enhancement of encapsulation efficiency as this value increased to 70%. The used technique is ionic gelation. It is a simple encapsulation technique that is based on the transformation of a dissolved polymer to a gel-like state. This is due to electrostatic interaction with the added polyanion. Obtained nanoparticles have spherical shape. Sizes ranged between 91 and 175 nm depending on investigated parameters. Overall *in vitro* release profile was similar to that obtained with the first study but the main advantage is the more rapid release. This could render *in vivo* application more possible. The prepared particles could present an interesting alternative for osteoporosis treatment by the oral route. I*n vivo* studies in rats were carried out to assess bioavailability and gastrointestinal tolerance of these particles.

Sommaire

Introduction générale

L'ostéoporose est la maladie métabolique la plus fréquente qui touche l'os. Elle se caractérise par une destruction micro-architecturale de la structure du tissu osseux. Ceci entraine une fragilisation de l'os et une augmentation du risque des fractures, particulièrement, au niveau des os longs et des vertèbres. Toutes les fractures qui sont liées à l'ostéoporose entraînent une forte morbidité. En plus, les fractures au niveau de la hanche et des vertèbres sont généralement associées à des taux élevés de mortalité. Ceci implique le grand danger que représente la maladie. Plusieurs approches thérapeutiques ont été développées pour le traitement de l'ostéoporose. Les bisphosphonates, les traitements hormonaux de substitution, les modulateurs sélectifs des récepteurs à œstrogènes, la calcitonine et la parathormone et ses analogues font partie des traitements pharmacologiques les plus utilisés. Cependant, ce sont généralement les bisphosphonates qui sont utilisés en première intention. Malgré leur grande efficacité, ces substances actives ne sont pas dénuées de limites en thérapeutique. En effet, elles ont une très faible biodisponibilité par la voie orale (<1%). Celle-ci est due principalement à un faible pouvoir de passage à travers la barrière gastro-intestinale. En plus, les bisphosphonates sont à l'origine de plusieurs effets indésirables qui se manifestent surtout au niveau œsophagien et gastro-intestinal. Ces effets se manifestent par des œsophagites, des ulcérations ou des vomissements. Ils sont dus à une irritation locale causée par un contact des cristaux des bisphosphonates avec la muqueuse œsophagienne et gastro-intestinale.

L'encapsulation est apparue comme une solution intéressante face aux problématiques de biodisponibilité et de toxicité des substances actives. Elle a été largement étudiée et appliquée durant les dernières décennies. Plusieurs substances actives (hydrophiles ou lipophiles) peuvent être encapsulées grâce à plusieurs techniques. Le choix d'une méthode particulière repose sur la nature physico-chimique de la substance active et du polymère en question. En effet, plusieurs polymères ont été développés pour servir de matrice pour les particules. Pour être administrable chez l'Homme, un polymère doit être biocompatible (c'est-à-dire qu'il ne provoque pas d'effets toxiques sur l'organisme humain) et biodégradable (c'est-à-dire dégradable en composés chimiques qui peuvent être facilement éliminés par l'organisme). Les polymères les plus utilisés sont les polyesters biodégradables comme l'acide polylactique (PLA), l'acide poly(lactique-co-glycolique) (PLGA) et la poly-ε-caprolactone (PCL). Selon leur taille, les particules obtenues seront soit des microparticules soit des nanoparticules. La

structure de ces véhicules se présente sous la forme de capsules (des vésicules de polymère à l'intérieur desquelles se trouve la substance active) ou de sphères (la substance active est dispersée à l'intérieur de la matrice du polymère). On parlera ainsi de microsphères ou nanosphères et de microcapsules ou nanocapsules. Les avantages de l'encapsulation sont multiples. Elle permet d'augmenter la stabilité de la substance active en la protégeant des facteurs environnementaux qui peuvent l'altérer. Ces facteurs peuvent être liés soit à l'environnement extérieur (par exemple : la lumière, l'humidité) ou à l'organisme humain (comme les enzymes). L'augmentation de la biodisponibilité est aussi un bénéfice majeur qui peut être obtenu suite à l'encapsulation. Celle-ci peut être attribuée à une meilleure biodistribution qui sera tributaire des propriétés physico-chimiques du polymère plutôt que de celles de la substance active. L'amélioration de la biodisponibilité peut être aussi obtenue grâce à un meilleur passage à travers les membranes. En plus, certaines particules furtives peuvent court-circuiter le système des phagocytes mononucléés en évitant la phagocytose. Ceci prolonge leur durée de résidence au niveau de la circulation sanguine. Par contre, pour d'autres vecteurs qui visent ce système, la phagocytose sera plutôt recherchée. L'efficacité sera donc augmentée dans ce cas. L'encapsulation permet aussi de protéger l'organisme des effets indésirables qui peuvent être engendrés par la molécule active. Par exemple, un passage à travers les membranes sous forme encapsulée protègera les muqueuses d'une éventuelle action irritante locale. La vectorisation d'une substance active peut être aussi atteinte. Cette propriété est conférée par la fixation d'un ligand à la surface des particules chargées en molécule active. Ce ligand peut se fixer sur un récepteur cellulaire ou tissulaire spécifique. L'encapsulation peut aussi assurer une libération prolongée grâce à la structure des polymères. Cette action est recherchée dans le cas de molécules qui nécessitent des prises multiples dans la journée. Ceci augmentera l'observance du patient et son confort surtout dans le cas des traitements chroniques. L'encapsulation permet aussi de masquer le goût désagréable de certaines molécules actives.

Dans ce contexte, plusieurs substances actives indiquées en ostéoporose ont été encapsulées dans des particules pour pallier les différents inconvénients qui sont liées à l'utilisation des formes pharmaceutiques conventionnelles. Le but était toujours de rechercher une augmentation de l'efficacité et une diminution de la toxicité ce qui permettra d'améliorer le rapport bénéfice/risque thérapeutique. On s'intéresse, dans notre projet de recherche, à une molécule active particulière, l'alendronate de sodium, qui appartient à la famille des bisphosphonates. L'alendronate de sodium possède un grand intérêt thérapeutique car il est

prescrit en première intention pour le traitement de l'ostéoporose. Cependant, il présente une faible biodisponibilité par voie orale (de l'ordre de 0,7%). Comme tous les bisphosphonates, beaucoup d'effets indésirables ont été relevés. Ils sont essentiellement dus à un contact local de l'alendronate avec la muqueuse œsophagienne et gastro-intestinale. L'alendronate sous forme de sel sodique a été développé pour remédier aux effets de la forme acide. Ainsi, les évènements indésirables sont devenus plus atténués mais continuent quand même à être observés. De ce fait, les patients qui prennent du Fosamax® (une forme commercialisée sous forme de comprimés) sont conseillés de prendre le comprimé avec un grand verre d'eau et de rester en position verticale (assise ou debout) pendant une période minimale de 30 minutes. Cette forme est aussi toujours déconseillée chez les patients souffrant d'anomalies au niveau gastro-intestinal. Notre but est d'encapsuler l'alendronate de sodium dans des nanoparticules à base d'un polymère biodégradable et biocompatible. Deux polymères seront étudiés à savoir, la poly-ε-caprolactone et le chitosane. Ces deux polymères ont été largement utilisés à des fins d'encapsulation de molécules actives. Les formes nanoparticules ont été choisies car elles n'ont pas été souvent développées pour encapsuler l'alendronate indiqué en ostéoporose. Les nanoparticules préparées visent à apporter un bénéfice thérapeutique en diminuant les effets indésirables et en augmentant la biodisponibilité.

Notre projet de recherche sera divisé en deux parties : une partie bibliographique et une partie expérimentale. La partie bibliographique servira de base pour le travail pratique. Elle consiste principalement en une étude de l'état de l'art en matière de techniques d'encapsulation et leurs applications récentes surtout pour le traitement de l'ostéoporose. Cette partie bibliographique sera divisée en trois parties : une partie sera dédiée aux techniques d'encapsulation basées sur les polymères préformés et leurs applications les plus récentes. La deuxième partie s'intéressera à une technique particulière, la nanoprécipitation, technique qui sera utilisée dans notre partie expérimentale. Une troisième partie traitera les différentes techniques d'encapsulation en ostéoporose et leurs applications.

La première partie bibliographique est consacrée à l'état de l'art en matière des dernières applications des méthodes d'encapsulation. Tout au long de cette partie, nous nous sommes particulièrement intéressés aux techniques qui sont basées sur l'utilisation des polymères préformés. Tout d'abord, une description des interactions polymère-solvant est fournie. Ensuite, les polymères les plus utilisés pour les applications pharmaceutiques sont relevés. Leurs différentes propriétés physico-chimiques sont aussi discutées. Par la suite, les différentes méthodes sont analysées en décrivant, pour chaque technique, le mode opératoire,

le mécanisme de formation des particules, les polymères les plus utilisés, les propriétés des molécules qui peuvent être encapsulées et les paramètres opératoires déterminants à maitriser. Pour chacune de ces techniques, les dernières applications sont présentées dans des tableaux récapitulatifs.

La nanoprécipitation, une méthode d'encapsulation largement utilisée, est traitée dans la deuxième partie bibliographique. Une explication du mécanisme de formation de nanoparticules par nanoprécipitation est discutée. Les avantages de la technique par rapport aux autres méthodes d'encapsulation sont aussi relevés. Les principales propriétés des phases aqueuses et organiques utilisées sont présentées. De même, les polymères les plus utilisés sont présentés. L'influence des conditions opératoires sur les propriétés des nanoparticules obtenues est aussi discutée. Toutes les approches innovantes en matière de nanoprécipitation, surtout en matière de dispositifs d'agitation, sont aussi traitées comme les dispositifs microfluidiques, l'émulsification par membrane et la nanoprécipitation flash. Enfin, les dernières applications *in vivo* de la technique ont été rapportées et discutées. La dernière partie bibliographique s'intéresse aux applications de l'encapsulation pour le traitement de l'ostéoporose. Les différentes molécules encapsulées et les différentes techniques utilisées pour leur encapsulation sont présentées. Les paramètres à maitriser pour le processus d'encapsulation sont aussi traités. Les différentes approches utilisées pour étudier la libération *in vitro* des substances actives sont présentées. Les techniques utilisées pour les études de biodistribution ou de tolérance *in vivo* ont été aussi étudiées. Les bénéfices *in vivo* qui ont été obtenus grâce l'encapsulation sont aussi discutés.

L'étude expérimentale comporte trois parties. La première partie consiste en une étude systématique de l'encapsulation de l'alendronate dans des nanoparticules à base de poly-ε-caprolactone. Deux techniques sont comparées : l'émulsion double et la nanoprécipitation. Les particules préparées sont aussi caractérisées. L'effet de plusieurs paramètres expérimentaux sur la taille, la charge de surface et le pourcentage d'encapsulation des nanoparticules est évalué. La libération *in vitro* de la substance active est aussi étudiée. Une analyse des données de libération de l'alendronate *in vitro* par des modèles mathématiques a été effectuée pour déterminer les mécanismes de libération de l'alendronate à partir des particules.

Dans la deuxième partie expérimentale, une autre technique d'encapsulation est utilisée qui est la gélification ionique. Le but recherché est d'optimiser d'une part le pourcentage

d'encapsulation de l'actif et d'autre part le profil de libération à partir des nanoparticules. Le polymère utilisé pour la préparation des particules est le chitosane. Une étude systématique de la technique est réalisée pour évaluer l'influence des paramètres opératoires. Enfin, une étude de la libération *in vitro* de la substance active est réalisée.

La troisième partie expérimentale consiste en une étude *in vivo* chez le rat. Cette partie comporte une étude de tolérance et une étude pharmacocinétique. Le but est de chercher un bénéfice thérapeutique qui pourrait être obtenu suite à une utilisation *in vivo* des nanoparticules de chitosane. Un tel bénéfice thérapeutique pourrait se manifester par une amélioration de la biodisponibilité et/ou par une diminution des effets indésirables et donc, une meilleure tolérance pour le traitement chronique.

Partie bibliographique

Introduction

Dans cette étude bibliographique, les données qui sont obtenues vont servir de base pour le travail expérimental. Le but de notre projet est d'encapsuler l'alendronate de sodium dans des nanoparticules polymériques. Nous cherchons aussi une éventuelle application *in vivo* de telles nanoparticules. Cette étude bibliographique permet de réaliser l'état de l'art de la recherche en matière d'encapsulation et de ses applications pharmaceutiques, notamment pour le traitement de l'ostéoporose. Une première partie traitera les différentes méthodes d'encapsulation en se limitant aux méthodes qui utilisent des polymères préformés. La deuxième partie consiste en une étude de l'état de l'art portant sur la technique de la nanoprécipitation. Le principe de la nanoprécipitation, ses dernières innovations et applications sont présentées et discutées. Dans la dernière partie, on s'intéressera plutôt aux techniques d'encapsulation qui ont été uniquement utilisées pour une application en ostéoporose.

La première étude permet de conclure que l'encapsulation a été appliquée pour plusieurs molécules actives avec une tendance actuelle d'encapsulation des molécules biologiques comme les ADN, ARN, plasmides et protéines. On observe aussi une tendance vers l'encapsulation de molécules indiquées en thérapie anticancéreuse. Plusieurs techniques d'encapsulation ont été utilisées. Pour chaque technique, le principe et les paramètres expérimentaux à maitriser sont décrits. Le principe général est de précipiter un polymère qui se trouve initialement en solution. Un état de l'art des polymères utilisés est réalisé. Les propriétés physicochimiques et les applications de ces polymères sont aussi présentées. Les polymères utilisés en encapsulation sont biodégradables et biocompatibles. Les polymères les plus utilisés sont les polyesters biodégradables.

Dans la partie consacrée à la nanoprécipitation, on constate que plusieurs études ont appliqué cette méthode simple et largement utilisée pour l'encapsulation de molécules hydrophobes ou hydrophiles en adoptant des approches différentes. L'étude a permis aussi de recenser les phases organiques et aqueuses les plus utilisées qui sont respectivement l'acétone et l'eau. Les polymères les plus utilisés sont toujours les polyesters biodégradables. La technique de nanoprécipitation flash présente particulièrement un grand intérêt. Elle permet, en effet, d'augmenter la reproductibilité et d'effectuer un contrôle sur les propriétés des nanoparticules

obtenues. Comme toutes les techniques d'encapsulation, l'application pharmaceutique la plus utilisée reste toujours la thérapie anticancéreuse en ciblant surtout la voie intraveineuse.

La dernière étude permet d'aborder les problématiques du traitement pharmacologique de l'ostéoporose à savoir, la faible solubilité et la biodisponibilité limitée des substances actives. L'encapsulation apparait comme une solution qui permet d'améliorer l'absorption de plusieurs molécules actives. L'étude montre aussi que les formes les plus préparées étaient des microparticules. Il s'avère aussi que les paramètres expérimentaux sont très importants et peuvent influer sur la libération *in vitro* et *in vivo* de la substance active et sur les paramètres pharmacocinétiques. Plusieurs bénéfices ont été obtenus grâce à l'encapsulation de molécules indiquées en ostéoporose. De tels bénéfices thérapeutiques ont été aussi confirmés *in vivo*.

Les particules préparées à partir de polymères préformés : préparation et applications

Publiée *sous forme d'une revue dans*

« EXCLI Journal »

EXCLI Journal *2014;13:28-57*

Les polymères biocompatibles et biodégradables sont largement utilisés pour l'encapsulation des molécules actives. Plusieurs types de particules ayant des caractéristiques différentes ont été préparés en adoptant une multitude de techniques. Ces systèmes protègent les substances actives de la dégradation, améliorent les propriétés biopharmaceutiques et peuvent assurer un ciblage actif ou passif ou bien une libération prolongée. De ce fait, leurs applications biologiques sont constamment en croissance. Une multitude de polymères est utilisée mais la grande majorité est biodégradable et biocompatible. Dans cette revue, on s'intéresse aux techniques qui se basent sur l'utilisation de polymères préformés. Tout d'abord, une description de l'interaction polymère-solvant est fournie. Ceci permet de comprendre les mécanismes engagés dans la formation de particules. La plupart des techniques exposées mettent en jeu une variation de la solubilité d'un polymère dans un solvant donné. La précipitation du polymère a lieu lorsqu'un non solvant est ajouté ou après une diminution rapide de sa solubilité dans un solvant déterminé. En effet, plusieurs paramètres peuvent influer sur la solubilité des polymères comme la nature du solvant, le pH, la salinité et la température du milieu. La solubilité des polyélectrolytes dans l'eau, par exemple, est hautement dépendante du pH et de la salinité. Les polymères les plus utilisés en encapsulation ont été rapportés et leurs propriétés physicochimiques ont été exposées. Il s'agit surtout de polymères biodégradables et biocompatibles. Un polymère biodégradable comporte dans sa structure une fonction labile comme la fonction ester ou amide par exemple. Une telle fonction peut être facilement dégradée par action enzymatique. Un polymère biocompatible présente une compatibilité avec les tissus biologiques. Ce sont surtout les polyesters biodégradables (comme les esters de l'acide lactique et glycolique), les polymères acryliques (comme Eudragit®) et les polymères naturels (chitosane et polydextranes) qui sont les plus utilisés en encapsulation. Pour chacun de ces polymères, les propriétés physicochimiques sont présentées et les principales applications pharmaceutiques sont exposées. Les propriétés d'un polymère déterminé peuvent avoir une répercussion sur le comportement *in vivo*. Par exemple, le chitosane présente des charges positives ce qui lui confère des propriétés mucoadhésives. De même, les propriétés gélifiantes des dextranes permettent aussi un contact prolongé avec les muqueuses. Pour l'acide poly(lactique-co-glycolique), le temps de dégradation varie selon le poids moléculaire. En plus, l'acide lactique étant plus hydrophobe que l'acide glycolique, le ratio des copolymères est aussi un paramètre important qui

intervient dans la dégradation du polymère. Pour chaque technique d'encapsulation, le mécanisme de la formation des particules est expliqué, les polymères utilisés sont rapportés et les paramètres expérimentaux à maitriser sont analysés et discutés. Pour la technique de nanoprécipitation, deux phases miscibles sont mises en jeu. La formation des nanoparticules a lieu suite à l'addition sous agitation magnétique d'un non solvant (généralement l'eau) à une solution du polymère dans un solvant organique. Les solvants organiques les plus utilisés sont l'éthanol et l'acétone. La phase aqueuse peut contenir un surfactant dont le rôle est d'augmenter la stabilité de la suspension des nanoparticules en empêchant leur agrégation. Les paramètres opérationnels à maitriser pour la technique sont le ratio phase organique/phase aqueuse, la concentration du polymère et du surfactant et la vitesse d'agitation. Une augmentation de la quantité du polymère entraine généralement une augmentation de la taille des particules. Une augmentation du volume de la phase aqueuse se traduit généralement par une diminution de la taille des particules. Une augmentation de la concentration du surfactant donne une diminution de la taille des particules. D'autre part, une augmentation de la vitesse d'agitation magnétique est accompagnée par une diminution de la taille. La nanoprécipitation est généralement utilisée pour l'encapsulation des molécules hydrophobes. Elle est utilisée principalement pour encapsuler des molécules anticancéreuses dans des nanoparticules à base de polyesters biodégradables. La technique émulsion-diffusion met en jeu 3 phases : une phase organique, une phase aqueuse et une phase de dilution. La phase organique est une solution de polymère dans un solvant organique partiellement miscible à l'eau comme l'acétate d'éthyle. La phase aqueuse est une solution de stabilisant dans l'eau et la phase de dilution consiste en un grand volume d'eau. Tout d'abord, la phase organique et la phase aqueuse sont saturées mutuellement jusqu'à l'obtention d'un équilibre thermodynamique. Ces deux phases sont ensuite émulsionnées à grande vitesse de dispersion pour obtenir une émulsion. La dernière étape est l'addition de la phase de dilution qui va entrainer une diffusion du solvant organique et donc, une précipitation du polymère sous forme de particules. En plus des paramètres décrits pour la nanoprécipitation, s'ajoutent d'autres qui peuvent influencer les propriétés des particules obtenues comme la température et le volume de la phase de dilution. La technique a été appliquée principalement pour l'encapsulation de molécules hydrophobes ayant des indications pharmaceutiques très variées. La technique d'émulsion simple est aussi largement utilisée pour l'encapsulation de molécules actives. Ici, c'est plutôt deux solvants immiscibles qui sont utilisés. La phase organique est généralement constituée par le dichlorométhane ou le chloroforme. La phase aqueuse est constituée généralement par une solution aqueuse d'un surfactant. En premier lieu, une émulsion de type

H/E (Huile dans l'eau) est préparée par une dispersion à grande vitesse. Une évaporation du solvant organique est ensuite réalisée pour obtenir la suspension des particules. Cette technique est utilisée généralement pour l'encapsulation de molécules hydrophobes dans les polymères de type polyesters biodégradables. La technique d'émulsion double, par contre, est bien adaptée aux molécules hydrophiles. Il s'agit de préparer une émulsion double qui est généralement de type E/H/E (eau dans l'huile dans l'eau). La phase aqueuse interne est une solution de la substance active. La phase organique est une solution de polymère. Elle peut aussi contenir une molécule active hydrophobe d'où l'intérêt de cette technique pour l'encapsulation de plus d'une substance active. Chacune des phases peuvent aussi contenir un stabilisant. La dispersion des différentes phases peut avoir lieu sous l'action d'un homogénéisateur ou des ultrasons. La technique d'émulsion double est surtout utilisée pour l'encapsulation de molécules hydrophiles principalement de nature biologique dans de l'acide poly(lactique-co-glycolique). La technique du spray drying a été aussi utilisée. L'intérêt de cette technique par rapport aux techniques citées précédemment réside dans le fait qu'elle se prête mieux à une transposition d'échelle. Pratiquement, une solution du polymère et de la substance active est pulvérisée dans une chambre chaude où elle va se transformer en gouttelettes et subir un séchage sous l'action de l'air chaud. Le polymère précipite ainsi en emprisonnant la substance active dans sa structure sous forme de particules. L'évaporation du solvant a lieu dans une période de temps très courte. Par conséquence, cette technique est intéressante pour les substances actives sensibles à la chaleur comme les protéines. Les paramètres à contrôler sont la température, l'humidité et le débit de l'air utilisé pour le séchage. Le processus de nébulisation ainsi que la nature de la solution sont des paramètres clés. La technique a été utilisée généralement pour l'encapsulation de molécules hydrophobes dans des microparticules à base de polyesters biodégradables. Les techniques de fluides supercritiques (surtout l'expansion rapide des solutions supercritiques) ont été récemment utilisées pour l'encapsulation de molécules actives. Ces techniques présentent l'avantage de ne pas utiliser de solvants organiques. L'état supercritique est atteint lorsque le fluide (généralement le CO_2) est ramené à des conditions de pression et de température en dessus de sa pression et de sa température critiques. Dans de telles conditions, le fluide aura la densité d'un liquide et par conséquent, un pouvoir solvant similaire à celui des liquides et, en même temps, des propriétés de transfert de masse comparables à celles d'un gaz. Pratiquement, la première étape de l'expansion rapide des solutions supercritiques consiste en une dissolution de la substance active et du polymère dans du CO_2 supercritique à l'intérieur d'une chambre à haute pression. Cette solution va passer à travers une buse ce qui résulte en une forte

diminution de la pression et donc, une précipitation du polymère et de la substance active sous forme de particules. Le recueil du produit se fait au niveau d'une unité d'extraction. Plusieurs paramètres peuvent influencer la taille des particules obtenues à savoir, la densité du fluide supercritique et le débit de la solution substance active-polymère. Comme les autres techniques, l'expansion rapide des solutions supercritiques a été surtout utilisée pour préparer des particules à base d'acide poly-lactique. La technique de gélification ionique est souvent utilisée avec les polymères naturels comme la gélatine, l'alginate, le chitosane et l'agarose. Cette méthode se base sur le passage d'un polymère de l'état soluté à l'état gel sous l'effet d'un refroidissement (comme pour la gélatine) ou de l'ajout d'un sel polyvalent (comme pour l'alginate et le chitosane). La gélification ionique est généralement appliquée pour la préparation de nanoparticules à base de chitosane contenant des molécules hydrophiles. D'autre part, la technique couche par couche se base sur une superposition de polyélectrolytes naturels ou synthétiques qui portent des charges opposées et forment des couches superposées grâce à une interaction électrostatique. Les applications récentes de chaque méthode décrite sont aussi présentées. Il s'avère que la plupart des molécules encapsulées sont des substances actives indiquées en thérapie anticancéreuse. Une deuxième catégorie de substances actives a été aussi largement étudiée à savoir, les molécules biologiques comme l'ADN, l'ARN, les plasmides et les protéines. Les caractéristiques des particules obtenues (taille et potentiel Zêta) sont présentées dans des tableaux récapitulatifs. Nous concluons ainsi que l'encapsulation a été largement appliquée dans le domaine biomédical. Le choix de la technique et du polymère est une étape cruciale qui dépend essentiellement des propriétés physicochimiques de la substance active. Les travaux de recherche les plus innovants utilisent un ciblage des particules préparées par un ligand qui va reconnaitre spécifiquement une cellule ou un tissu particulier. On constate aussi une tendance vers le développement de vecteurs intelligents grâce aux « matériaux stimulables ». Ces derniers libèrent la substance active suite à une variation de température (ce qui est intéressant par exemple en cas d'infections) ou de pH (exemple du diabète). Un intérêt particulier a été aussi prêté aux techniques qui n'utilisent pas de solvants organiques comme la méthode l'expansion rapide des fluides supercritiques. Les techniques qui assurent une bonne reproductibilité et qui permettent une transposition d'échelle plus simple bénéficient aussi d'un grand intérêt dans les travaux de recherche. Ceci est, par exemple, le cas d'émulsification par membrane. De telles méthodes sont fortement intéressantes pour une application industrielle.

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

Review article:

PARTICLES FROM PREFORMED POLYMERS AS CARRIERS FOR DRUG DELIVERY

K. Miladi[a,b], D. Ibraheem[a], M. Iqbal[a], S. Sfar[b], H. Fessi[a], A. Elaissari[a]*

[a] University of Lyon, F- 69622, Lyon, France; Lyon 1 University, Villeurbanne, CNRS, UMR 5007, Laboratoire d'Automatique et de Génie des Procédés, LAGEP-CPE-308G, 43 bd. du 11 Nov.1918, F-69622, Villeurbanne, France
[b] University of Monastir, Laboratoire de pharmacie galénique, Rue Avicenne, 5000 Monastir, Tunisia

* Corresponding author: Phone: +33-472431841, Fax: +33-472431682
E-mail: elaissari@lagep.univ-lyon1.fr

ABSTRACT

Biodegradable and biocompatible polymers are widely used for the encapsulation of drug molecules. Various particulate carriers with different sizes and characteristics have been prepared by miscellaneous techniques. In this review, we reported the commonly used preformed polymer based techniques for the preparation of micro and nano-structured materials intended for drug encapsulation. A description of polymer-solvent interaction was provided. The most widely used polymers were reported and described and their related research studies were mentioned. Moreover, principles of each technique and its crucial operating conditions were described and discussed. Recent applications of all the reported techniques in drug delivery were also reviewed.

Keywords: Drug delivery, particles, polymer, encapsulation, carriers, operating conditions

INTRODUCTION

Particulate carriers have gained tremendous interest during the last decades which permitted to deliver many hydrophilic and hydrophobic molecules. Obtained particles present small size which facilitates their absorption. These drug delivery systems protect active pharmaceutical ingredients from degradation, enhance biopharmaceutical properties and could provide passive or active targeting or sustained delivery. Biomedical applications of the developed carriers are continuously growing (Ahmad, 2013; Soares, 2013; Miladi et al., 2013). Although, they present different physicochemical properties, the used polymers are mainly biocompatible and biodegradable. A multitude of techniques are used to obtain these particles. These methods differ by their principles and the nature of drug molecules that could be encapsulated. Some successfully marketed products led to an enlargement of the applications and the interest given by researchers to these drug delivery systems. Choice of the technique and operating conditions is crucial to obtain formulations bearing good properties for *in vitro* and *in vivo* applications. In this review, we will focus on polymeric particles and give a scope about the most used polymers. We will also describe the common preformed polymer based techniques used

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

for the encapsulation of drug molecules. We will also review the major applications of the developed particles during the last years and their main properties.

1. POLYMER-SOLVENT INTERACTIONS

Many techniques that rely on preformed polymers have been used for the preparation of particulate carriers. Although these methods are quite different, they generally share a unique principle which is polymer precipitation. Precipitation of the polymer occurs either when a non solvent is added or after subsequent decrease of its solubility in a solvent. Many parameters could influence polymer solubility such as, solvent nature, pH, salinity and temperature of the dispersion medium. Solubility of polyelectrolytes in water, for example, is highly pH and salinity dependent (Gennes, 1979), while that of poly(alkyl acrylamide) and poly(alkyl methacrylamide), is mainly temperature dependent (Elaissari, 2002). In fact, nanoprecipitation and emulsion based techniques are based on the addition of a non solvent to the polymer which causes its precipitation. However, ionic gelation technique, for instance, in which generally a polyelectrolyte is used as polymer, is based on the addition of a salt or an oppositely charged polymer. This results in a change in the salinity of the medium and the appearance of electrostatic interactions and thus, leads to polymer precipitation. The thermodynamic behavior of the polymer in a given solution is highly dependent on the Flory χ-parameter. This parameter is defined as the free energy change per solvent molecule (in k_BT units) when a solvent-solvent contact is shifted to a solvent-polymer contact. It is expressed by the following mathematical equations:

$$\chi = \frac{\Delta G}{k_B T} = \frac{\Delta H - T\Delta S}{k_B T} = \frac{1}{2} - A\left(1 - \frac{\theta}{T}\right)$$

Equation (1)

where kB and T are Boltzmann constant and temperature, respectively; A and θ parameters are defined as follows:

$$A = \frac{2\Delta S + k_B}{2k_B}$$

Equation (2)

$$\theta = \frac{2\Delta H}{2\Delta S + k_B}$$

Equation (3)

It can be seen that the A parameter is directly related to entropy changes, whereas θ temperature is a function of both entropic and enthalpic variations. When θ temperature = T, the corresponding Flory χ-parameter = 1/2, at which the second Virial coefficient is equal zero (Elias, 2003). The latter can be easily determined from light scattering measurements of a diluted polymer solution. At θ temperature conditions, the binary interactions among constituents will be negligible and only the excluded volume effects will be predominant. Consequently, the solvent will be a good solvent for the polymer when $\chi < 1/2$ and a poor one when $\chi > 1/2$ (Minost et al., 2012).

2. COMMONLY USED POLYMERS FOR ENCAPSULATION

Several polymers have been used for drug encapsulation but only biodegradable and biocompatible ones are suitable for biomedical applications. The biodegradability of a polymer is acquired by the presence of a labile function such as ester, orthoester, anhydride, carbonate, amide, urea or urethane in their backbone. These polymers could be of natural (polysaccharides and protein based polymers) or synthetic (polyesters) nature (Pillai and Panchagnula, 2001). The most commonly used polymers for drug encapsulation are polyesters (lactide and glycolide copolymers, poly-ε-caprolactone), acrylic polymers (polymethacrylates) and polyamides (gelatin and albumin). The selection of the right polymer is a crucial step to obtain particles that are suitable for a well-defined application. In fact, polymers' structures are highly differ-

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

ent and their surface and bulk properties are highly relevant for the obtaining of the desirable biological application. Copolymers could be also used to monitor the hydrophobicity of the materials. Some polymers are poly(ethyleneglycol) (PEG) copolymerized in order to decrease nanoparticle recognition by the reticular endothelial system. Table 1 contains examples of the most used biocompatible and biodegradable polymers in encapsulation. Some polymers, especially those having mucoadhesive properties, could also be used for coating the nanocarriers (Mazzaferro et al., 2012; Zandanel and Vauthier, 2012).

Table 1: Commonly used polymers

Materials	References
Polymers	
Natural polymers	
Chitosan	Elmizadeh et al., 2013; Fàbregas et al., 2013; Khalil et al., 2012; Konecsni et al., 2012; Du et al., 2009; Bernkop-Schnürch et al., 2006; Gan et al., 2005; Asada et al., 2004
Dextran	Liang et al., 2013; Dai et al., 2012; Sajadi Tabassi et al., 2008; Koten et al., 2003
Dextran derivatives	Kanthamneni et al., 2012; Kauffman et al., 2012; Aumelas et al., 2007; Miyazaki et al., 2006
Cyclodextrins	Çirpanli et al., 2009; Memişoğlu et al., 2003; Pariot et al., 2002; Lemos-Senna et al., 1998
Gelatin	Nahar et al., 2008; Balthasar et al., 2005; Vandervoort and Ludwig, 2004; Bruschi et al., 2003
Synthetic polymers	
Biodegradable polyesters	
PLGA	Gyulai et al., 2013; Beck-Broichsitter et al., 2012; Morales-Cruz et al., 2012; Beck-Broichsitter et al., 2011; Nehilla et al., 2008; Song et al., 2008; Budhian et al., 2007; Bozkir and Saka, 2005; Fonseca et al., 2002;Yang et al., 1999; Govender et al., 1999
PLA	Bazylińska et al., 2013; Fredriksen and Grip 2012; Kadam et al., 2012; Kumari et al., 2011; Ataman-Önal et al., 2006; Lamalle-Bernard et al., 2006; Hyvönen et al., 2005; Katare et al., 2005; Chorny et al., 2002; Leo et al., 2000
PCL	Behera and Swain, 2012; Guerreiro et al., 2012; Hernán Pérez de la Ossa et al., 2012; Khayata et al., 2012; Arias et al., 2010; Wang et al., 2008; Limayem Blouza et al., 2006; Tewa-Tagne et al., 2006; Yang et al., 2006; Le Ray et al., 2003; Chawla and Amiji 2002; Raval et al., 2011; Hombreiro Pérez et al., 2000; Benoit et al., 1999; Masson et al., 1997
Poly(lactide-co-glycolide-co-caprolactone)	Zhang et al., 2006
Acrylic polymers	
Eudragit	Hao et al., 2013; Das et al., 2010; Eidi et al., 2010; Trapani et al., 2007; Galindo-Rodríguez et al., 2005; Haznedar and Dortunç 2004; Pignatello et al., 2002
Others	
Polyvinylbenzoate	Labruère et al., 2010
Pegylated polymers	
Chitosan-PEG	Seo et al., 2009
MPEG-PCL	Falamarzian and Lavasanifar, 2010; Xin et al., 2010
PCL-PEG-PCL	Suksiriworapong et al., 2012; Huang et al., 2010; Gou et al., 2009
Poly(caprolactone)-poly(ethylene oxide)-polylactide	Hu et al., 2003
PLA-PEG	Sacchetin et al., 2013; Essa et al., 2010; Ishihara et al., 2010; Vila et al., 2005; Vila et al., 2004; Govender et al., 2000; Huang et al., 1997
PLA-PEG-PLA	Chen et al., 2011; Ruan and Feng 2003
MPEG-PLA	Zheng et al., 2010; Dong and Feng, 2007; Dong and Feng, 2004

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

2.1 Natural polymers

2.1.1 Chitosan

Chitosan is obtained by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (crabs, shrimp, etc.) and cell walls of fungi. It is a cationic and biodegradable polysaccharide consisting of repeating D-glucosamine and N-acetyl-D-glucosamine units, linked via (1-4) glycosidic bonds. Chitosan is non toxic and can be digested in the physiological environment, either by lysozymes or by chitinases, which are present in the human intestine and in the blood. These properties led to increased interest for this polymer in pharmaceutical research and industry as a carrier for drug delivery (Mao et al., 2010). In addition, chitosan has mucoadhesive properties owing to its positive charge that allows interaction with the negatively-charged mucosal surface. Consequently, the use of chitosan as a matrix (Patil and Sawant, 2011) or as a coating material (Mazzarino et al., 2012) in drug encapsulation had become a promising strategy to prolong the residence time, to increase the absorption of active molecules through the mucosa (Mao et al., 2010; Alpar et al., 2005) and also for targeted delivery (Park et al., 2010).

2.1.2 Dextran and its derivatives

Dextran polymers are produced by bacteria from sucrose. Chemical synthesis is also possible. These glucose polymers consist predominantly of linear α-1,6-glucosidic linkage with some degree of branching via 1,3-linkage. Dextran-based microspheres have got much attention because of their low toxicity, good biocompatibility and biodegradability, which are of interest for application in biomedical and pharmaceutical fields (Mehvar, 2000). Many detxran polymers such as Sephadex® (cross-linked dextran microspheres) as well as Spherex® (cross-linked starch microspheres) were used as carriers for drug delivery. Other derivatives of dextran and starch including diethyl aminoethyl dextran and polyacryl starch have also been used for mucosal drug delivery. Illum et al. (2001) proposed some mechanisms to explain absorption enhancement effects of cross-linked starch and dextran microspheres intended to nasal delivery which are: (1) Deposition of the microspheres in the less or non ciliated anterior part of the nasal cavity and slower nasal clearance; (2) Retention of the formulation in the nasal cavity for an extended time period because of the bioadhesive properties of the microspheres and (3) The local high drug concentration provided by the gelled system in close contact with the epithelial absorptive surface (Illum et al., 2001).

2.1.3 Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides that contain at least six D-(+) glucopyranose units which are attached by α-(1,4) glucosidic bonds. They have been widely used for the formulation of drugs with bioavailability concerns resulting from poor solubility, poor stability and severe side effects. There are 3 natural CDs which are α-, β-, and γ-CDs (with 6, 7, or 8 glucose units respectively) (Challa et al., 2005). In addition, amphiphilic cyclodextrins are synthetic derivatives of natural cyclodextrins. Such derivatives are able to self-organize in water to form micelles and nano-aggregates, which is interesting for pharmaceutical applications, mainly, encapsulation (Gèze et al., 2002). In fact, amphiphilic cyclodextrins have recently been used to prepare nanoparticles and nanocapsules without surfactants and have shown high drug-loading capacity with favorable release properties (Lemos-Senna et al., 1998; Çirpanli et al., 2009; Duchêne, 1999). They have also been used for targeting and for increasing drug loading (Duchêne et al., 1999).

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

2.1.4 Gelatin

Gelatin is a natural polymer that is derived from collagen. It is commonly used for pharmaceutical and medical applications because of its biodegradability and biocompatibility in physiological environments. Gelatin is attractive for use in controlled release due to its nontoxic, bioactive properties and inexpensive price. It is also a polyampholyte having both cationic and anionic groups along with hydrophilic groups. Mechanical properties, swelling behavior and thermal properties of gelatin depend significantly on its crosslinking degree (Young et al., 2005).

2.2 Biodegradable polyesters

Polyester-based polymers are among of the most widely investigated materials for drug delivery. Poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymers poly(lactic acid-co-glycolic acid) (PLGA) along with poly-ε-caprolactone are some of the well-defined biomaterials with regard to design and performance for drug-delivery applications.

2.2.1 PLGA

PLGA, a copolymer of lactic acid and glycolic acid, has generated tremendous interest due to its excellent biocompatibility, biodegradability, and mechanical strength. PLGA is approved by the US FDA and European Medicine Agency (EMA) in various drug delivery systems in humans. In order to improve the formulation of controlled drug delivery systems, an understanding of the physical, chemical, and biological properties of polymers is helpful. In fact, the polymer is commercially available with different molecular weights and copolymer compositions. The degradation time can vary from several months to several years, depending on the molecular weight and copolymer ratio (Danhier et al., 2012). For example, lactic acid is more hydrophobic than glycolic acid and, therefore, lactide-rich PLGA copolymers are less hydrophilic, absorb less water, and subsequently, degrade more slowly (Dinarvand et al., 2011). PLGA particles are widely used to encapsulate active molecules with a broad spectrum of pharmaceutical applications (Danhier et al., 2012; Menei et al., 2005; Singh et al., 2004).

2.2.2 PLA

PLA is a biocompatible and biodegradable synthetic polyester which undergoes scission in the body to monomeric units of lactic acid. The latter is a natural intermediate in carbohydrate metabolism. PLA possess good mechanical properties and it is largely used for the preparation of particles (Gupta and Kumar, 2007).

2.2.3 PCL

It was in 1930s that the ring-opening polymerization of PCL was studied. The biodegradable property of this synthetic polymer was first identified in 1973. PCL is suitable for controlled drug delivery due to its high permeability to many drugs and non-toxicity (Sinha et al., 2004). Molecular weight dependent surface hydrophobicity and crystallinity of PCL are the causes for its slower biodegradation in two distinct phases such as random non-enzymatic cleavage and enzymatic fragmentation. Lipophilic drugs are generally distributed uniformly in the matrix while hydrophilic drugs tend to move towards the interface and remain on the surface of PCL formulation in adsorbed state. Diffusion was described as the only possible mechanism by which the lipophilic drugs release from PCL particles as they were shown to be intact for a much longer duration *in vivo*. However, two phenomena could be implicated in hydrophilic drugs' release. Highly lipophilic drugs that resist complete diffusion are released upon surface erosion by enzymatic action while hydrophilic drugs that accumulate at the interface during the formulation processes are released by desorption at the initial period of release study

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

or dosage intake. This results in a biphasic drug release pattern for PCL particles with much higher burst release for hydrophilic drugs than lipophilic ones (Dash and Konkimalla, 2012).

2.3 Pegylated polymers

Many of the above cited polymers could be conjugated to PEG chains, which allows the enhancement of their hydrophilicity and permits the obtaining of a stealth surface that could protect the prepared carriers from degradation by the cells belonging to the reticuloendothelial system. Conjugation to PEG confers also bioadhesive properties for the carriers (Yoncheva et al. 2005).

3. Used methods for the encapsulation of active molecules

3.1 Nanoprecipitation

The nanoprecipitation technique was first developed by Fessi et al. in 1986 (Devissaguet et al., 1991). The technique allows the obtaining of either nanospheres or nanocapsules. The organic phase could be added to the aqueous phase under magnetic stirring. This one-step process allows the instantaneous and reproducible formation of monodisperse nanoparticles. Nanoprecipitation is simple, is by far the fastest, most reproducible, and industrially feasible preparation procedure of nanospheres (Vauthier and Bouchemal, 2009). Practically, two miscible phases are required: an organic solvent in which the polymer is dissolved and an aqueous phase (non-solvent of the polymer). The most common used organic solvents are ethanol and acetone. Such solvents are miscible in water and easy to remove by evaporation. Some oils could be added to these solvents to allow the dissolving of the active (Rosset et al., 2012). As Figure 1 shows, the method is based on the addition of one phase to the other under moderate magnetic stirring which causes the interfacial deposition of a polymer after displacement of the organic solvent from the organic solution. This leads to the formation of a suspension of nanoparticles. The organic phase could be a mixture of solvents such as, mixture of acetone with water or ethanol etc. Similarly, the aqueous phase could consist of a mixture of non-solvents and could contain surfactants. Commonly used polymers are biodegradable polyesters, especially PCL, PLA and PLGA (Rao and Geckeler, 2011). Particle formation process includes three basic steps which are, particle nucleation, molecular growth and aggregation. The rate of every step has a crucial impact on the particle size distribution. Supersaturation is the driving force that manages all of these steps, namely, particles nucleation rate. Supersaturation, itself, is influenced by fluid dynamics and mixing. In fact, low stirring rate results in low nucleation rates while higher mixing rates give high nucleation rates (Lince et al., 2008).

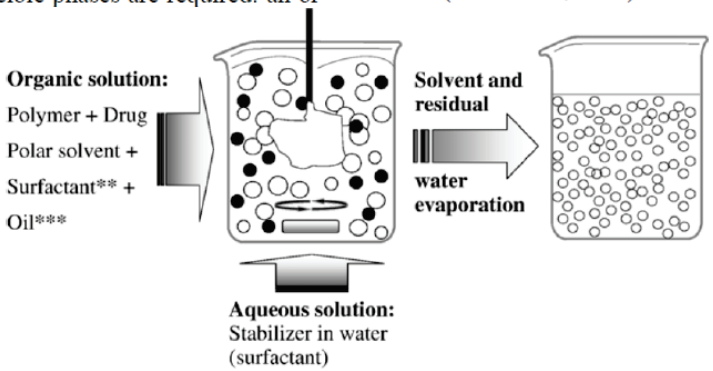


Figure 1: The nanoprecipitation technique (Pinto Reis et al., 2006)

Operational parameters that should be controlled include the organic phase to non organic phase ratio, the concentration of the polymer and the stabilizer and the amount of the drug. Every one of these parameters may exert an impact on the characteristics of the obtained nanoparticles (size, uniformity and charge). In fact, an increase of the polymer amount generally increases particles' size (Chorny et al., 2002; Simşek et al., 2013; Dong and Feng, 2004; Asadi et al., 2011). The same effect was obtained after increasing the polymer molecular weight (Limayem Blouza et al., 2006; Holgado et al., 2012). These findings were explained by an increase of the viscosity of the organic phase which rendered solvent diffusion more difficult and thus, led to larger nanoparticles' size. The effect of increasing organic phase volume seems conflicting: some studies showed that it causes a decrease of the particles size (Dong and Feng, 2004) while others showed the opposite phenomenon (Asadi et al., 2011). Increasing the water phase amount leads to a decrease of the particles size as a result of the increased diffusion of the water-miscible solvent in the aqueous phase and thus, the more rapid precipitation of the polymer and formation of nanoparticles (Budhian et al., 2007). An increase of the surfactant amount generally causes a decrease of the particles size and reduces size distribution (Contado et al., 2013; Siqueira-Moura et al., 2013). Some studies did not, however, found significant change following surfactant amount increase (Dong and Feng, 2004). The nature of the surfactant may also influence the particles' size (Limayem Blouza et al., 2006). Increasing mixing rate decreases the particles size as it causes faster diffusion rate (Asadi et al., 2011). Theoretical drug loading may also influence particles size and drug loading (Govender et al., 1999). Nanoprecipitation is generally designed for the encapsulation of hydrophobic drug molecules (Seju et al., 2011; Katara and Majumdar, 2013; Seremeta et al., 2013). Such actives may be dissolved within the organic phase. Bilalti et al. (2005) described a nanoprecipitation technique intended to the encapsulation of hydrophilic molecules but the size of the obtained particles was not sufficiently uniform (Bilati et al., 2005). To further improve the reproducibility of the nanoprecipitation technique and make it more convenient for industrial applications, membrane contactor and microfluidic technology were successfully used (Khayata et al., 2012; Xie and Smith, 2010). These techniques allow better size control within different batches of particles. Table 2 contains some examples of the applications of the nanoprecipitation technique in drug delivery during the last years. It can be concluded that polyesters are among the most used polymers for the preparation of the nanoparticles by this technique.

3.2 Emulsion diffusion (ESD)

ESD was first developed by Quintanar-Guerrero and Fessi (Quintanar-Guerrero et al., 1996) to prepare PLA based nanospheres. Three liquid phases are needed in this technique: an organic phase, an aqueous phase and a dilution phase. The organic phase generally contains the polymer and the hydrophobic drug. The aqueous phase is a solution of a stabilizing agent while the dilution phase usually consists of a large volume of water. Mutual saturation of the aqueous and organic phase allows further obtaining of a thermodynamically equilibrated emulsion upon high speed homogenization. Subsequent addition of an excess of water enables the diffusion of the organic solvent from the dispersed phase resulting in precipitation of the polymer and the formation of the particles (Figure 2). Commonly used polymers in this method include PCL, PLA and Eudragit® (Mora-Huertas et al., 2010). Table 3 shows that the technique is mainly used for the encapsulation of hydrophobic molecules. However, hydrophilic molecules may also be encapsulated by a modified solvent diffusion method using an aqueous inner phase (Ma

et al., 2001). Operating conditions affecting the size of the obtained particles include external/internal phase ratio, emulsification stirring rate, volume and temperature of water for dilution, polymer amount and concentration of the stabilizer (Quintanar-Guerrero et al., 1996; Mora-Huertas et al., 2010). Influence of high shear homogenization and sonication on the particles size was assessed and it was found that sonication was more efficient for particle size reduction. The nature of the surfactant influenced also the particles size. In fact, when Pluronic F68 (PF68), didodecyldimethylammonium bromide (DMAB) and polyvinylalcohol (PVA) were compared, DMAB gave the smallest particles but with the lowest encapsulation efficiency (Jain et al., 2011). Particles size was also described to increase with an increase of initial drug amount (Youm et al., 2012), polymer amount (Youm et al., 2012; Esmaeili et al., 2011) and the oil phase volume (Esmaeili et al., 2011; Poletto et al., 2008). An increase of the surfactant amount resulted in a decrease of the size but it seems that above some level further significant size reduction is no longer possible (Jain et al., 2011; Surassmo et al., 2010). An increase of the homogenization rate led to a decrease of the particles' size (Jain et al., 2011; Kwon et al., 2001; Galindo-Rodríguez et al., 2005). Likely, the same effect was obtained following an increase of the temperature and the volume of added water (Kwon et al., 2001; Song et al., 2006). The nature of the organic solvent also influenced particle size (Song et al., 2006). Table 3 shows some of the recent applications of the ESD technique.

Table 2: Applications of the nanoprecipitation technique

Encapsulated molecule	Polymer	Size (nm)	Zeta potential (mV)	Reference
Doxorubicin	Gelatin-co-PLA-DPPE	131.5-161.1	-	Han et al., 2013
Aceclofenac	Eudragit RL 100	75.5-184.4	22.5 - 32.6	Katara and Majumdar, 2013
Doxorubicin	Dextran-b-polycaprolactone	95-123.3	-	Li et al., 2013
Chloroaluminum phthalocyanine	PLGA	220.3-326.3	-17.7-(-40.9)	Siqueira-Moura et al., 2013
Efavirenz	PCL and Eudragit® RS 100	89.5 - 173.9	-17.9-53.8	Seremeta et al., 2013
Paclitaxel	PLGA	50 - 150	-15 - (-20)	Wang et al., 2013
Retinoic acid	PLA	153.6-229.8	-27.4-(-20.9)	Almouazen et al., 2012
Brimonidine Tartrate	Eudragit® RL 100	123.5 - 140.2	13.1- 20.8	Khan et al., 2012
Vitamin E	PCL	123-320	-24.5-(-1.46)	Khayata et al., 2012
Paclitaxel	Hydrophobized pullulan	127.6-253		Lee et al., 2012
Curcumin	PCL, chitosan	104-125	(-0.099)-79.8	Mazzarino et al., 2012
Diclofenac	PCL	152	-50	Mora-Huertas et al., 2012
Amphotericin B	PLGA	86-153	-31.4-(-9.1)	Van de Ven et al., 2012
Epirubicin	Poly(butyl cyanoacrylate)	217-235	-4.5-(-0.1)	Yordanov 2012
Camptothecin	Beta-cyclodextrin PLGA PCL	281 187 274	-13 -0.06 -19	Cırpanlı et al., 2011
Naringenin	Eudragit® E	90	-	Krishnakumar et al., 2011
Olanzapine	PLGA	91.2	-23.7	Seju et al., 2011

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

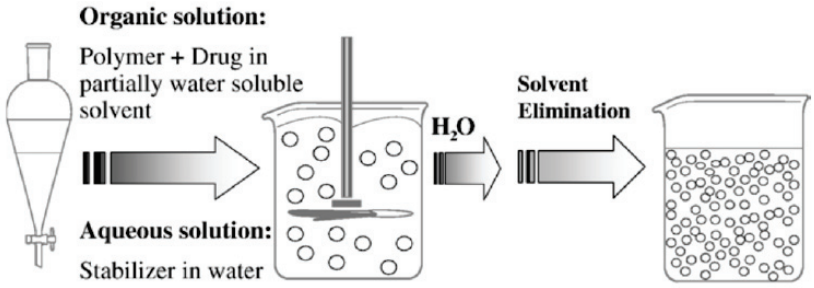


Figure 2: Emulsion diffusion technique (Pinto Reis et al., 2006)

Table 3: Applications of the emulsion diffusion method

Encapsulated molecule	Polymer	Size (µm)	Zeta potential (mV)	Reference
Articaine	PCL	-	-	Campos et al., 2013
Omeprazole	Eudragit L 100-55	0.256.3- 0.337	8.92 - 16.53	Hao et al., 2013
Curcumin	Polyurethane and polyurea	0.216- 4.901	-	Souguir et al., 2013
Matricaria recutita L. extract	PEG-PBA-PEG	0.186- 0.446	-	Esmaeili et al., 2011
Bovine serum albumin	Chitosan	81-98	-	Karnchanajindanun et al., 2011
Alendronate	PLGA	0.145	-4.7	Cohen-Sela et al., 2009
An oligonucleo-tide	PLA	0.390	-	Delie et al., 2001

3.3 Simple Emulsion evaporation (SEE)

The SEE technique is widely used in the field of particulate carriers' development. This method was first developed by (Vanderhoff et al., 1979). It consists on the formation of a simple emulsion followed by the evaporation of the organic solvent. Subsequent precipitation of the polymer allows the obtaining of the particles (Figure 3). Practically, for oil in water emulsion method, the polymer is dissolved in a volatile and non miscible organic solvent such as chloroform, ethylacetate or dichloromethane. This organic phase, in which the drug and the polymer are dissolved, is then dispersed by high speed homogenization or by sonication in an aqueous phase containing a surfactant. Once an oil-in-water (o/w) emulsion is obtained, the evaporation of the organic solvent permits the precipitation of the polymer and thus, the formation of the particles. As it can be seen in Table 4, SEE is generally used for the encapsulation of hydrophobic drugs (O'Donnell and McGinity, 1997). The evaporation of the organic solvent is obtained by moderately stirring the emulsion at room temperature or under high temperature and low pressure conditions. The obtained particles can be then harvested by ultracentrifugation or filtration, then washed and lyophilized. Membrane technology was also used to prepare particles by the simple emulsion technique (Doan et al., 2011). Another alternative of the technique is the use of water in oil emulsion method that is suitable for the encapsulation of hydrophilic active molecules. Particulate carriers are obtained after evap-

oration of the water phase which causes the precipitation of the hydrophilic polymer (Banerjee et al., 2012). Parameters that have to be managed include organic phase to water phase ratio, nature of the surfactant and its concentration, stirring rate, polymer amount and evaporation rate. Decreasing the organic solvent volume resulted generally in a decrease of particle size (Budhian et al., 2007). Particle size could also be decreased by increasing surfactant amount (Valot et al., 2009; Manchanda et al., 2010; Khaled et al., 2010; Su et al., 2009), increasing stirring rate (Su et al., 2009; Lee et al., 2012; Avachat et al., 2011; Yadav and Sawant, 2010) or increasing aqueous phase volume (Adibkia et al., 2011). However, an increase of polymer amount generally increases particles' size (Doan et al., 2011; D'Aurizio et al., 2011; Adibkia et al., 2011; Agnihotri and Vavia, 2009). Table 4 shows the applications of the SEE technique in drug delivery. Polyesters were widely used for the encapsulation of hydrophobic drugs.

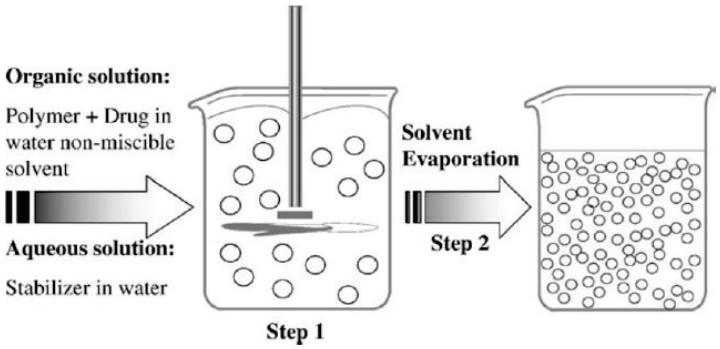


Figure 3: Simple emulsion solvent evaporation (Pinto Reis et al., 2006)

Table 4: Applications of simple emulsion solvent evaporation technique

Encapsulated molecule	Polymer	Size (µm)	Zeta potential (mV)	Reference
Curcumin	PLGA and PLGA-PEG	0.161-0.152	-	Khalil et al., 2013
Efavirenz	PCL and Eudragit® RS 100	0.083-0.219	53	Seremeta et al., 2013
Human amylin	PCL	0.202	-	Guerreiro et al., 2012
Azithromycin	PLGA	14.11-15.29	-	Li et al., 2012
Teniposide	PLGA	0.113-0.135	-36.6-(-23.1)	Mo et al., 2012
Camptothecin	PCL-PEG-PCL	4.2-5.4	-	Dai et al., 2011
Naproxen	PLGA	352-824	-	Javadzadeh et al., 2010
Doxorubicin	PLGA	0.137-0.164	-12.3-(-9.9)	Manchanda et al., 2010
Dexamethasone	PLGA	5.18-7	-	Rawat and Burgess, 2010
Ibuprofen	Eudragit RSPO	14-51.1	-	Valot et al., 2009

3.4 Double emulsion evaporation (DEE)

Double emulsion technique is suitable for the encapsulation of hydrophilic molecules (see Table 5 and Figure 4). Generally, the method consists on the dispersion of an aqueous phase in a non miscible organic solvent to form the first emulsion (W1/O). This dispersion is performed under high shear homogenization or low power sonication for a short time. This step is followed by the dispersion of the obtained emulsion in a second aqueous phase containing a hydrophilic emulsifier. Again, homogenization could be carried under high shear homogenization or with a sonication probe. When sonication is used, it must be performed at low power and within a short period of time to not break the first emulsion (Giri et al., 2013). After the formation of the multiple emulsion, evaporation of the volatile organic solvent under low pressure (by a rotary evaporator) or at ambient temperature allows the obtaining of the particulate carriers (Figure 4). There are other types of multiple emulsions like w/o/o or o/w/o (Giri et al., 2013). A lot of parameters may influence the properties of the obtained particles such as, relative phases' ratio (Khoee et al., 2012), amount of the polymer, its nature and molecular weight (Zambaux et al., 1998; Péan et al., 1998; Van de Ven et al., 2011), nature of the surfactants and their amounts (Zhao et al., 2007; Khoee and Yaghoobian, 2009; Dhanaraju et al., 2004), homogenization speed (Eley and Mathew, 2007; Basarkar et al., 2007), the composition of the external phase (Péan et al., 1998; Tse et al., 2009) and evaporation speed (Khoee et al., 2012). Operating conditions may also influence strongly encapsulation efficiency (Tse et al., 2009; Billon et al., 2005; Silva et al., 2013; Zhou et al., 2013; Karataş et al., 2009; Hachicha et al., 2006; Al-Kassas, 2004; Cun et al., 2011; Gaignaux et al., 2012; Cun et al., 2010). Membrane technique and microfluidic devices were also used to prepare particulate carriers by the DES method (Vladisavljević and Williams, 2008; van der Graaf et al., 2005).

3.5 Spray drying

Spray drying is a simple process which gained too much interest due to its cost-effectiveness and scalability (Sou et al., 2011). Practically, a polymer containing drug solution is atomized and sprayed into a drying chamber where droplets are dried by heated air (See Figure 5). Reduction of droplets' size that follows atomization allows the obtaining of an enormous surface area between droplets and the drying gas. The subsequent precipitation of the polymer permits the encapsulation of the drug within the obtained particulate carriers. The evaporation of the solvent occurs within a very short period of time. Consequently, the materials never reach the inlet temperature of drying gas. This is very attractive for encapsulating heat-sensitive drug molecules like proteins (Cal and Sollohub, 2010; Sollohub and Cal, 2010; Prata et al., 2013). Many operating conditions could influence the properties of the obtained particles. Parameters to be controlled include the drying air temperature and humidity (Bruschi et al., 2003), the rate and fluid dynamics of the air flow, the atomization process (Droplet size, spray rate, spray mechanism) and the composition of ingredients and excipients in the feeding solution (Rattes and Oliveira, 2007). PLA (Baras et al., 2000; Gander et al., 1996; Sastre et al., 2007; Muttil et al., 2007), PLGA (Wang and Wang, 2002; Mu and Feng, 2001; Castelli et al., 1998; Bittner et al., 1999; Prior et al., 2000; Conti et al., 1997), PCL, methacrylate polymers (Esposito et al., 2002; Año et al., 2011; Cruz et al., 2010; Hegazy et al., 2002; Raffin et al., 2008) and chitosan (He et al., 1999; Giunchedi et al., 2002; Cevher et al., 2006) are among the most used polymers in spray-drying method. As Table 6 shows, the technique allowed the obtaining of mainly microparticles bearing better drug solubility and sustained release.

Table 5: Applications of the double emulsion technique

Encapsulated molecule	Polymer	Size (µm)	Zeta potential (mV)	Reference
Vancomycin	PLGA	0.450-0.466	-7.2-(-3.5)	Zakeri-Milani et al., 2013
Prostaglandin E1	PLGA	7-22.5	-	Gupta and Ahsan, 2011
Deoxyribonuclease I	PLGA	0.190-0.349	-	Osman et al., 2011
S. equi antigens	PCL	0.242-0.450	-53.1-38.7	Florindo et al., 2009
Hepatitis B surface antigen	PLGA	1-5	0.51-14	Thomas et al., 2009
Plasmid DNA	PLGA	1.9-4.6	-24.6-(-22.9)	Tse et al., 2009

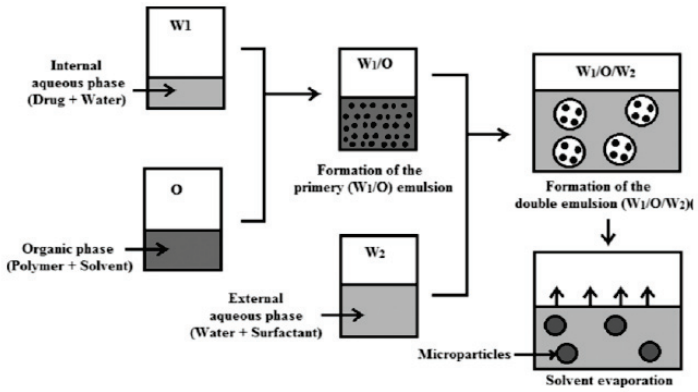


Figure 4: Double emulsion solvent evaporation technique (Giri et al., 2013)

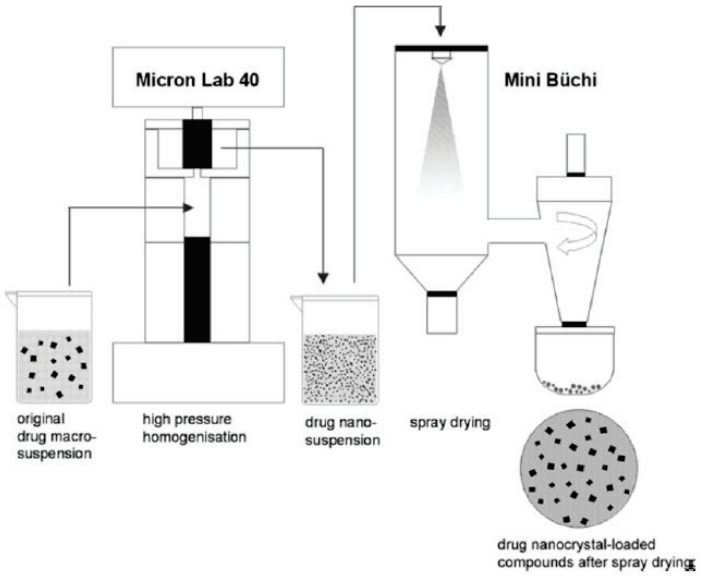


Figure 5: The spray drying method (Pinto Reis et al., 2006)

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

Table 6: Applications of the spray drying technique

Encapsulated molecule	Polymer	Size (µm)	Zeta potential (mV)	Reference
Nimodipine	PLGA	1.9-2.37	-	Bege et al., 2013
Theophylline	Eudragit RS30D	< 60	-	Garekani et al., 2013
Ofloxacin	PLA	2.6-4.9	-	Palazzo et al., 2013
Sodium diclofenac	PGA-co-PDL PEG-PGA-co-PDL and mPEG-co-(PGA-co-PDL)	2.3 3.9 2.5	-32.2 -29.9 -31.2	Tawfeek, 2013
Sodium fluoride	Chitosan	3.4-5.3	-	Keegan et al., 2012
Plasmid	Chitosan	2.5-11.7	-	Mohajel et al., 2012
Heparin	PLGA	2.5-3.8	-63.5 - (-28.2)	Yildiz et al., 2012
Alendronate	Eudragit® S100	13.8	-	Cruz et al., 2010
Zolmitriptan	Chitosan glutamate and Chitosan base	2.6-9.4	-	Alhalaweh et al., 2009
Triamcinolone	PLGA	0.5-1.5	-	da Silva et al., 2009
Acyclovir	Chitosan	18.7-34.9	-	Stulzer et al., 2009

3.6 Supercritical fluid technology (SFT)

In the recent years, novel particle formation techniques using supercritical fluids (SCF) have been developed in order to overcome some of the disadvantages of conventional techniques that are: (1) poor control of particle size and morphology; (2) degradation and lost of biological activity of thermo sensitive compounds; (3) low encapsulation efficiency and (4) low precipitation yield (Santos et al., 2013). Moreover, SFT presents the main advantage of not requiring the use of toxic solvents. In fact, SCF based technologies have attracted enormous interest for the production of microparticles and nanoparticles (Table 7), since their emergence in the early 1990s (Sanli et al., 2012).

The supercritical state is achieved when a substance is exposed to conditions above its critical pressure and temperature. In such conditions, the fluid will have liquid-like density and, thus, solvating properties that are similar to those of liquids and, at the same time, gas-like mass transfer properties. Carbon dioxide (CO_2) is the most commonly used critical fluid. In fact, CO_2 is nontoxic, nonflammable and easy recyclable. Moreover, CO_2 has moderate critical parameters of CO_2 (a critical pressure of 7.4 MPa and a critical temperature of 304.1 K) and low price and is highly available which makes it very attractive from an economical point of view and also for the processing of labile compounds (Elizondo et al., 2012). Supercritical fluid technology methods can be divided in four methods which are rapid expansion of supercritical solution (RESS), Particles from gas saturated solutions (PGSS), gas antisolvent (GAS) and supercritical antisolvent process (SAS). These methods depend on whether CO_2 was used as a solvent, a solute or an antisolvent. Figure 6 shows the experimental set up of the RESS technique. In the RESS technique, the drug and the polymer are first dissolved in supercritical CO_2 in high pressure chamber. The subsequent passing of the solution through a nozzle results in a rapid decrease of the pressure and thus, a precipitation of the drug particles embedded in the polymer matrix and their recovery in the extraction unit (Byrappa et al., 2008). Many parameters such as the density of the SCF (Pressure and temperature of supercritical fluid) (Kalani and Yunus, 2012), flow rate of drug-polymer solution and/or CO_2 and formulation variables (Martin et al., 2002) could influence the size of the obtained particles. Table 7 shows that SFT was used for

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

the processing of nanoparticles and microparticles mainly based on polyesters.

3.7 Ionic gelation (IG)

IG method is used mainly with natural hydrophilic polymers to prepare particulate carriers. These polymers include gelatin, alginate, chitosan and agarose. IG has the advantage of not using organic solvents. The technique is based on the transition of the polymer from liquid state to a gel (Figure 7). For instance, gelatin based particles are obtained after the hardening of the droplets of emulsified gelatin solution. The particles are obtained after cooling gelatin emulsion droplets below the gelation point in an ice bath. For alginate, however, particles are produced by drop-by-drop extrusion of the sodium alginate solution into the calcium chloride solution. Sodium alginate is, in fact, a water-soluble polymer that gels in the presence of multivalent cations such as calcium. Chitosan particles are prepared by spontaneous formation of complexes between the positively charged chitosan and polyanions (tripolyphosphate or gelatin) or by the gelation of a chitosan solution dispersed in an oil emulsion (Mahapatro and Singh, 2011). Figure 7 illustrates the gelation mechanism of polysaccharides. At high temperatures, a random coil conformation is assumed. With decreasing temperature, the aggregation of double helices structure forms the physical junctions of gels (Rees and Welsh, 1977). Table 8 displays some recent applications of IG. This technique has been mainly used to prepare chitosan nanoparticles.

Table 7: Applications of the SCF technology

Encapsulated molecule	Polymer	Size (µm)	Zeta potential (mV)	Reference
Hydrocortisone acetate	PLGA	1-5	-	Falco et al., 2013
17α-methyltestosterone	PLA	5.4-20.5	13.9 - 67.7	Sacchetin et al., 2013
Paracetamol	PLA	0.301-1.461	-	Kalani and Yunus, 2012
5-fluorouracil	PLLA-PEG/PEG	0.175	-	Zhang et al., 2012
Human growth hormone	PLGA	93	-	Jordan et al., 2010
Azacytidine	PLA	2	-	Argemí et al., 2009
Bovine serum albumin	PLA	2.5	-	Kang et al., 2009
Retinyl palmitate	PLA	0.040-0.110	-	Sane and Limtrakul, 2009
Indomethacin	PLA	2.35	-	Kang et al., 2008

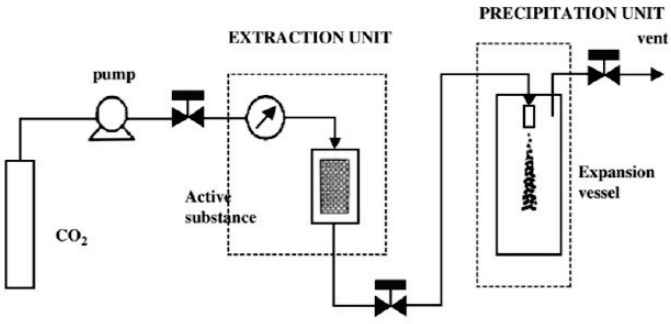


Figure 6: Schematic presentation of the experimental set up for the RESS process (Byrappa et al., 2008)

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

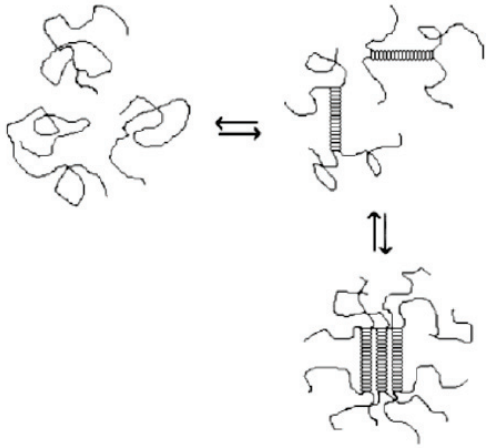


Figure 7: Gelation mechanism of polysaccharides in water (Guenet, 1992)

Table 8: Some applications of the ionic gelation technique

Encapsulated molecule	Polymer	Size (µm)	Zeta potential (mV)	Reference
Articaine hydrochloride	Alginate/chitosan	0.340-0.550	-22 - (-19)	de Melo et al., 2013
TNF-α siRNA	Trimethyl chitosan-cysteine	0.146	25.9	He et al., 2013
Paclitaxel	O-carboxymethyl chitosan	0.130-0.180	-30 - (-12)	Maya et al., 2013
pDNA	Chitosan	0.153-0.403	46.2-56.9	Cadete et al., 2012
Gemcitabine	Chitosan	0.095	-	Derakhshandeh and Fathi, 2012
Dexamthasone sodium phosphate	Chitosan	0.256-0.350	-	Doustgani et al., 2012
Itraconazole	Chitosan	0.190-0.240	11.5-18.9	Jafarinejad et al., 2012
5-fluorouracil and leucovorin	Chitosan	0.040-0.097	25.6-28.9	Li et al., 2011
Insulin	Chitosan and arabic gum	0.172-0.245	35.7-43.4	Avadi et al., 2010
CKS9 peptide sequence	Chitosan	0.226	-	Yoo et al., 2010

3.8 Layer by layer

Polyelectrolyte self assembly is also called layer-by-layer (LbL) assembly process. The earliest technology was based on the assembly of colloidal particles on a solid core (Iler, 1966). From the 1990s, applications were expanded. LbL allowed, in fact, the assembly of polyelectrolyte films using biopolymers, proteins, peptides, polysaccharides and DNA (Powell et al., 2011). This approach was first developed by Sukhorukov et al. (Sukhorukov et al., 1998). Polyelectrolytes are classified according to their origin. Standard synthetic polyelectrolytes include poly(styrene sulfonate) (PSS), poly (dimethyldiallylammonium chloride) (PDDA), poly(ethylenimine) (PEI), poly(N-isopropyl acrylamide (PNIPAM), poly-

(acrylic acid) (PAA), poly (methacrylic acid) (PMA), poly(vinyl sulfate) (PVS) and poly(allylamine) (PAH). Natural polyelectrolytes include nucleic acids, proteins and polysaccharides such as, alginic acid, chondroitin sulfate, DNA, heparin, chitosan, cellulose sulfate, dextran sulfate and carboxymethylcellulose (de Villiers et al., 2011). The obtained particles are vesicular and are called polyelectrolyte capsules. Assembly process is based on irreversible electrostatic attraction that leads to polyelectrolyte adsorption at supersaturating polyelectrolyte concentrations. Other interactions such as, hydrogen bonds, hydrophobic interactions and Van der Waals forces were also described (de Villiers et al., 2011). A colloidal template that serves to the adsorption of the polyelectrolyte is also needed. The commonly used cores for the formulated particles are derived from stabilized colloidal dispersions of charged silica, charged poly(styrene) spheres, metal oxides, polyoxometalates and conducting liquid crystalline polymers. Carrier systems can be functionalized with stimuli-responsive components that respond to temperature, pH and ionic strength. The polymers/colloids used in LbL technique can also be functionalized to alter their properties preceding layer by layer assembly. Experimental parameters that have to be managed include coating material concentration, ion concentration and the pH of the medium (Vergaro et al., 2011). Polymer assembly occurs after incubation of the template in the polymer solution or by decreasing polymer solubility by drop-wise addition of a miscible solvent (Radtchenko et al., 2002). This procedure could be repeated with a second polymer to allow sequential deposition of multiple polymer layers (Figure 8). LbL presents advantages over several conventional coating methods: (1) simplicity of the process and equipment; (2) its suitability for coating most surfaces; (3) availability and abundance of natural and synthetic colloids; (4) flexible application to objects with irregular shapes and sizes; (5) formation of stabilizing coats and (6) control over the required multilayer thickness (de Villiers et al., 2011). Table 9 contains some recent applications of LbL technique.

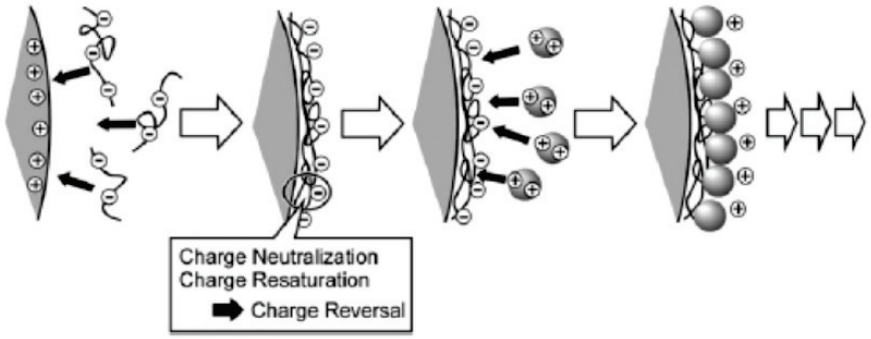


Figure 8: The layer by layer technique based on electrostatic interaction (Ariga et al., 2011)

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

Table 9: Applications of the layer-by-layer technique

Active	Polyelectrolytes	Size (µm)	Zeta potential (mV)	References
Kaempferol	Sodium Alginate and protamine sulfate	0.161	- 8.9	Kumar et al., 2012
Designed peptide DP-2015	Poly-l-glutamic acid and poly-l-lysine	-	-	Powell et al., 2011
5-fluorouracil	Poly(L-glutamic acid) and chitosan	1	25-40	Yan et al., 2011
Plasmid DNA	Plasmid DNA and reducible hyperbranched poly(amidoamine) or polyethylenimine	-	-	Blacklock et al., 2009
Artemisinin	Alginate, gelatin and chitosan	0.806	-33	Chen et al., 2009
Insulin	Glucose oxidase and catalase	6	-	Qi et al., 2009
Heparin	Poly(styrene sulfonate) and chitosan	1	-10.4	Shao et al., 2009
Acyclovir	Poly(vinyl galactose ester-co-methacryloxyethyl trimethylammonium chloride) and poly(sodium 4-styrenesulfonate)	-	-	Zhang et al., 2008a
Propranolol hydrochloride	Poly(vinyl galactose ester-*co*-methacryloxyethyl trimethylammonium chloride) and Poly(sodium 4-styrenesulfonate)	5-15.6	-	Zhang et al., 2008b

CONCLUSION

Encapsulation of active molecules is a crucial approach that has been widely used for many biomedical applications. It permits enhancement of bioavailability of molecules, sustained delivery, passive or active targeting and decrease of toxicity and side effects. These formulations can render some active molecules more suitable for a specific route such as the delivery of proteins by the oral route or the delivery of some drugs via the blood brain barrier. Thus, they enhance efficiency, patient compliance and allow successful management of diseases. Many biodegradable and biocompatible polymers were investigated. The choice of the technique and the suitable polymer is a crucial step. It depends on the physicochemical properties of the drug to be encapsulated. The management of operating conditions is also a hard task to monitor particles' properties and to enhance drug loading. Recent research works are focusing on active targeting by the coating the carriers by biomolecules that specifically recognize a well-defined cell receptor. One can also notice a shift for more 'intelligent' drug delivery systems. Responsive materials, for example, react to a specific physiological stimulus such as a variation of pH to release the encapsulated drug. Other thermo-sensitive materials deliver drugs at a specific temperature. It can be noted also that more attention is paid to safer methods that avoid the use of organic solvents (RESS) or to techniques that provide better reproducibility and easy scalability (microfluidics and membrane emulsification technology), which could be attractive for industrial processing.

REFERENCES

Adibkia K, Javadzadeh Y, Dastmalchi S, Mohammadi G, Niri FK, Alaei-Beirami M. Naproxen-eudragit rs100 nanoparticles: preparation and physicochemical characterization. Colloids Surf B Biointerfaces 2011;83:155–9.

Agnihotri SM, Vavia PR. Drug loaded poly[lac(glc-leu)] microparticles: formulation and release characteristics. Colloids Surf B Biointerfaces 2009;74: 336–9.

Ahmad H. A special issue on polymer and hybrid particles for biomedical applications. J Colloid Sci Biotechnol 2013;2:153–4.

Al-Kassas R. Design and in vitro evaluation of gentamicin-eudragit microspheres intended for intraocular administration. J Microencapsul 2004;21:71–81.

Alhalaweh A, Andersson S, Velaga SP. Preparation of zolmitriptan-chitosan microparticles by spray drying for nasal delivery. Eur J Pharm Sci 2009;38: 206–14.

Almouazen E, Bourgeois S, Boussaïd A, Valot P, Malleval C et al. Development of a nanoparticle-based system for the delivery of retinoic acid into macrophages. Int J Pharm 2012;430:207–15.

Alpar HO, Somavarapu S, Atuah KN, Bramwell VW. Biodegradable mucoadhesive particulates for nasal and pulmonary antigen and dna delivery. Adv Drug Deliv Rev 2005;57:411–30.

Añó G, Esquisabel A, Pastor M, Talavera A, Cedré B et al. A new oral vaccine candidate based on the microencapsulation by spray-drying of inactivated vibrio cholerae. Vaccine 2011;29:5758–64.

Argemí A, Vega A, Subra-Paternault P, Saurina J. Characterization of azacytidine/poly(l-lactic) acid particles prepared by supercritical antisolvent precipitation. J Pharm Biomed Anal 2009;50:847–52.

Arias JL, López-Viota M, Sáez-Fernández E, Ruiz MA. Formulation and physicochemical characterization of poly(ε-caprolactone) nanoparticles loaded with ftorafur and diclofenac sodium. Colloids Surf B Biointerfaces 2010;75:204–8.

Ariga K, Lvov YM, Kawakami K, Ji Q, Hill JP. Layer-by-layer self-assembled shells for drug delivery. Adv Drug Deliv Rev 2011;63:762–71.

Asada M, Takahashi H, Okamoto H, Tanino H, Danjo K. Theophylline particle design using chitosan by the spray drying. Int J Pharm 2004; 270:167–74.

Asadi H, Rostamizadeh K, Salari D, Hamidi M. Preparation of biodegradable nanoparticles of triblock pla-peg-pla copolymer and determination of factors controlling the particle size using artificial neural network. J Microencapsul 2011;28:406–16.

Ataman-Önal Y, Munier S, Ganée A, Terrat C, Durand P-Y et al. Surfactant-free anionic pla nanoparticles coated with hiv-1 p24 protein induced enhanced cellular and humoral immune responses in various animal models. J Control Release 2006;112:175–85.

Aumelas A, Serrero A, Durand A, Dellacherie E, Leonard M. Nanoparticles of hydrophobically modified dextrans as potential drug carrier systems. Colloids Surf B Biointerfaces 2007;59:74–80.

Avachat AM, Bornare PN, Dash RR. Sustained release microspheres of ropinirole hydrochloride: effect of process parameters. Acta Pharm Zagreb Croat 2011;61:363–76.

Avadi MR, Sadeghi AMM, Mohammadpour N, Abedin S, Atyabi F et al. Preparation and characterization of insulin nanoparticles using chitosan and arabic gum with ionic gelation method. Nanomed Nanotechnol Biol Med 2010;6:58–63.

Balthasar S, Michaelis K, Dinauer N, von Briesen H, Kreuter J, Langer K. Preparation and characterisation of antibody modified gelatin nanoparticles as drug carrier system for uptake in lymphocytes. Biomaterials 2005;26:2723–32.

Banerjee P, Deb J, Roy A, Ghosh A, Chakraborty P. Fabrication and development of pectin microsphere of metformin hydrochloride. ISRN Pharm 2012;230621. doi: 10.5402/2012/230621.

Baras B, Benoit M-A, Gillard J. Parameters influencing the antigen release from spray-dried poly(dl-lactide) microparticles. Int J Pharm 2000;200:133–45.

Basarkar A, Devineni D, Palaniappan R, Singh J. Preparation, characterization, cytotoxicity and transfection efficiency of poly(dl-lactide-co-glycolide) and poly(dl-lactic acid) cationic nanoparticles for controlled delivery of plasmid dna. Int J Pharm 2007;343:247–54.

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

Bazylińska U, Lewińska A, Lamch Ł, Wilk KA. Polymeric nanocapsules and nanospheres for encapsulation and long sustained release of hydrophobic cyanine-type photosensitizer. Colloids Surf Physicochem Eng Asp 2013.

Beck-Broichsitter M, Ruppert C, Schmehl T, Guenther A, Betz T et al. Biophysical investigation of pulmonary surfactant surface properties upon contact with polymeric nanoparticles in vitro. Nanomed Nanotechnol Biol Med 2011;7:341–50.

Beck-Broichsitter M, Schweiger C, Schmehl T, Gessler T, Seeger W, Kissel T. Characterization of novel spray-dried polymeric particles for controlled pulmonary drug delivery. J Control Release 2012; 158:329–35.

Bege N, Renette T, Endres T, Beck-Broichsitter M, Hänggi D, Kissel T. In situ forming nimodipine depot system based on microparticles for the treatment of posthemorrhagic cerebral vasospasm. Eur J Pharm Biopharm 2013;84:99–105.

Behera T, Swain P. Antigen adsorbed surface modified poly-ε-caprolactone microspheres stimulates both adaptive and innate immune response in fish. Vaccine 2012;30:5278–84.

Benoit MA, Baras B, Gillard J. Preparation and characterization of protein-loaded poly(epsilon-caprolactone) microparticles for oral vaccine delivery. Int J Pharm 1999;184:73–84.

Bernkop-Schnürch A, Heinrich A, Greimel A. Development of a novel method for the preparation of submicron particles based on thiolated chitosan. Eur J Pharm Biopharm 2006;63:166–72.

Bilati U, Allémann E, Doelker E. Development of a nanoprecipitation method intended for the entrapment of hydrophilic drugs into nanoparticles. Eur J Pharm Sci 2005;24:67–75.

Billon A, Chabaud L, Gouyette A, Bouler J-M, Merle C. Vancomycin biodegradable poly(lactide-co-glycolide) microparticles for bone implantation. influence of the formulation parameters on the size, morphology, drug loading and in vitro release. J Microencapsul 2005;22:841–52.

Bittner B, Mäder K, Kroll C, Borchert H-H, Kissel T. Tetracycline-hcl-loaded poly(dl-lactide-co-glycolide) microspheres prepared by a spray drying technique: influence of γ-irradiation on radical formation and polymer degradation. J Control Release 1999; 59:23–32.

Blacklock J, You Y-Z, Zhou Q-H, Mao G, Oupický D. Gene delivery in vitro and in vivo from bioreducible multilayered polyelectrolyte films of plasmid dna. Biomaterials 2009;30:939–50.

Bozkir A, Saka OM. Formulation and investigation of 5-fu nanoparticles with factorial design-based studies. Il Farm 2005;60:840–6.

Bruschi M, Cardoso ML, Lucchesi M, Gremião MP. Gelatin microparticles containing propolis obtained by spray-drying technique: preparation and characterization. Int J Pharm 2003;264:45–55.

Budhian A, Siegel SJ, Winey KI. Haloperidol-loaded plga nanoparticles: systematic study of particle size and drug content. Int J Pharm 2007;336: 367–75.

Byrappa K, Ohara S, Adschiri T. Nanoparticles synthesis using supercritical fluid technology - towards biomedical applications. Adv Drug Deliv Rev 2008; 60:299–327.

Cadete A, Figueiredo L, Lopes R, Calado CCR, Almeida AJ, Gonçalves LMD. Development and characterization of a new plasmid delivery system based on chitosan-sodium deoxycholate nanoparticles. Eur J Pharm Sci 2012;45:451–8.

Cal K, Sollohub K. Spray drying technique. i: hardware and process parameters. J Pharm Sci 2010;99: 575–86.

Campos EVR, de Melo NFS, de Paula E, Rosa AH, Fraceto LF. Screening of conditions for the preparation of poly(-caprolactone) nanocapsules containing the local anesthetic articaine. J Colloid Sci Biotechnol 2013;2:106–11.

Castelli F, Conti B, Maccarrone DE, Conte U, Puglisi G. Comparative study of 'in vitro' release of anti-inflammatory drugs from polylactide-co-glycolide microspheres. Int J Pharm 1998;176:85–98.

Cevher E, Orhan Z, Mülazımoğlu L, Şensoy D, Alper M et al. Characterization of biodegradable chitosan microspheres containing vancomycin and treatment of experimental osteomyelitis caused by methicillin-resistant staphylococcus aureus with prepared microspheres. Int J Pharm 2006;317:127–35.

Challa R, Ahuja A, Ali J, Khar RK. Cyclodextrins in drug delivery: an updated review. AAPS Pharm Sci Tech 2005;6:E329–57.

Chawla JS, Amiji MM. Biodegradable poly(ε-caprolactone) nanoparticles for tumor-targeted delivery of tamoxifen. Int J Pharm 2002;249:127–38.

Chen A-Z, Zhao Z, Wang S-B, Li Y, Zhao C, Liu Y-G. A continuous ress process to prepare pla–peg–pla microparticles. J Supercrit Fluids 2011;59:92–7.

Chen Y, Lin X, Park H, Greever R. Study of artemisinin nanocapsules as anticancer drug delivery systems. Nanomed Nanotechnol Biol Med 2009;5: 316–22.

Chorny M, Fishbein I, Danenberg HD, Golomb G. Lipophilic drug loaded nanospheres prepared by nanoprecipitation: effect of formulation variables on size, drug recovery and release kinetics. J Control Release 2002;83:389–400.

Çirpanli Y, Bilensoy E, Lale Doğan A, Çaliş S. Comparative evaluation of polymeric and amphiphilic cyclodextrin nanoparticles for effective camptothecin delivery. Eur J Pharm Biopharm 2009; 73:82–9.

Cırpanlı Y, Allard E, Passirani C, Bilensoy E, Lemaire L et al. Antitumoral activity of camptothecin-loaded nanoparticles in 9l rat glioma model. Int J Pharm 2011;403:201–6.

Cohen-Sela E, Chorny M, Koroukhov N, Danenberg HD, Golomb G. A new double emulsion solvent diffusion technique for encapsulating hydrophilic molecules in plga nanoparticles. J Control Release 2009;133:90–5.

Contado C, Vighi E, Dalpiaz A, Leo E. Influence of secondary preparative parameters and aging effects on plga particle size distribution: a sedimentation field flow fractionation investigation. Anal Bioanal Chem 2013;405:703–11.

Conti B, Bucolo C, Giannavola C, Puglisi G, Giunchedi P, Conte U. Biodegradable microspheres for the intravitreal administration of acyclovir: in vitro/in vivo evaluation. Eur J Pharm Sci 1997;5: 287–93.

Cruz L, Assumpção E, Andrade SF, Conrado DJ, Kulkamp IC et al. Gastroresistant microparticles containing sodium alendronate prevent the bone loss in ovariectomized rats. Eur J Pharm Sci 2010;40: 441–7.

Cun D, Foged C, Yang M, Frokjaer S, Nielsen HM. Preparation and characterization of poly(dl-lactide-co-glycolide) nanoparticles for sirna delivery. Int J Pharm 2010;390:70–5.

Cun D, Jensen DK, Maltesen MJ, Bunker M, Whiteside P et al. High loading efficiency and sustained release of sirna encapsulated in plga nanoparticles: quality by design optimization and characterization. Eur J Pharm Biopharm 2011;77:26–35.

D'Aurizio E, van Nostrum CF, van Steenbergen MJ, Sozio P, Siepmann F et al. Preparation and characterization of poly(lactic-co-glycolic acid) microspheres loaded with a labile antiparkinson prodrug. Int J Pharm 2011;409:289–96.

Da Silva AA Jr, de Matos JR, Formariz TP, Rossanezi G, Scarpa MV et al. Thermal behavior and stability of biodegradable spray-dried microparticles containing triamcinolone. Int J Pharm 2009;368:45–55.

Dai C, Wang Y, Hou X. Preparation and protein adsorption of porous dextran microspheres. Carbohydr Polym 2012;87:2338 43.

Dai M, Xu X, Song J, Fu S, Gou M et al. Preparation of camptothecin-loaded pcec microspheres for the treatment of colorectal peritoneal carcinomatosis and tumor growth in mice. Cancer Lett 2011;312:189–96.

Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A, Préat V. Plga-based nanoparticles: an overview of biomedical applications. J Control Release 2012;161:505–22.

Das S, Suresh PK, Desmukh R. Design of eudragit rl 100 nanoparticles by nanoprecipitation method for ocular drug delivery. Nanomed Nanotechnol Biol Med 2010;6:318–23.

Dash TK, Konkimalla VB. Poly-ε-caprolactone based formulations for drug delivery and tissue engineering: a review. J Control Release 2012;158: 15–33.

De Melo NFS, Campos EVR, de Paula E, Rosa AH, Fraceto LF. Factorial design and characterization studies for articaine hydrochloride loaded alginate/chitosan nanoparticles. J Colloid Sci Biotechnol. 2013;2:146–52.

De Villiers MM, Otto DP, Strydom SJ, Lvov YM. Introduction to nanocoatings produced by layer-by-layer (lbl) self-assembly. Adv Drug Deliv Rev 2011; 63:701–15.

Delie F, Berton M, Allémann E, Gurny R. Comparison of two methods of encapsulation of an oligonucleotide into poly(d,l-lactic acid) particles. Int J Pharm 2001;214:25–30.

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

Derakhshandeh K, Fathi S. Role of chitosan nanoparticles in the oral absorption of gemcitabine. Int J Pharm 2012;437:172–7.

Devissaguet J-P, Fessi H, Puisieux F. Process for the preparaton of dispersible colloidal systems of a substance in the form of nanocapsules. US Patent 5049322, 1991.

Dhanaraju MD, Jayakumar R, Vamsadhara C. Influence of manufacturing parameters on development of contraceptive steroid loaded injectable microspheres. Chem Pharm Bull (Tokyo) 2004;52:976–9.

Dinarvand R, Sepehri N, Manoochehri S, Rouhani H, Atyabi F. Polylactide-co-glycolide nanoparticles for controlled delivery of anticancer agents. Int J Nanomed 2011;6:877–95.

Doan TVP, Couet W, Olivier JC. Formulation and in vitro characterization of inhalable rifampicin-loaded plga microspheres for sustained lung delivery. Int J Pharm 2011;414:112–7.

Dong Y, Feng SS. Methoxy poly(ethylene glycol)-poly(lactide) (mpeg-pla) nanoparticles for controlled delivery of anticancer drugs. Biomaterials 2004;25: 2843–9.

Dong Y, Feng SS. In vitro and in vivo evaluation of methoxy polyethylene glycol–polylactide (mpeg–pla) nanoparticles for small-molecule drug chemotherapy. Biomaterials 2007;28:4154–60.

Doustgani A, Farahani EV, Imani M, Doulabi AH. Dexamethasone sodium phosphate release from chitosan nanoparticles prepared by ionic gelation method. J Colloid Sci Biotechnol 2012;1:42–50.

Du W-L, Niu S-S, Xu Y-L, Xu Z-R, Fan C-L. Antibacterial activity of chitosan tripolyphosphate nanoparticles loaded with various metal ions. Carbohydr Polym 2009;75:385–9.

Duchêne D. Cyclodextrins in targeting application to nanoparticles. Adv Drug Deliv Rev 1999;36:29–40.

Duchêne D, Ponchel G, Wouessidjewe D. Cyclodextrins in targeting: application to nanoparticles. Adv Drug Deliv Rev 1999;36:29–40.

Eidi H, Joubert O, Attik G, Duval RE, Bottin MC et al. Cytotoxicity assessment of heparin nanoparticles in nr8383 macrophages. Int J Pharm 2010;396:156–65.

Elaissari A. Thermally sensitive latex particles. In: Birdi K (ed.): Handbook of surface and colloid chemistry, 2nd ed. Boca Raton, FL: CRC Press, 2002.

Eley JG, Mathew P. Preparation and release characteristics of insulin and insulin-like growth factor-one from polymer nanoparticles. J Microencapsul 2007; 24:225–34.

Elias H-G. An introduction to plastics. Weinheim: Wiley-VCH, 2003.

Elizondo E, Veciana J, Ventosa N. Nanostructuring molecular materials as particles and vesicles for drug delivery, using compressed and supercritical fluids. Nanomedicine 2012;7:1391–1408.

Elmizadeh H, Khanmohammadi M, Ghasemi K, Hassanzadeh G, Nassiri-Asl M, Garmarudi AB. Preparation and optimization of chitosan nanoparticles and magnetic chitosan nanoparticles as delivery systems using box–behnken statistical design. J Pharm Biomed Anal 2013;80:141–6.

Esmaeili A, Saremnia B, Koohian A, Rezazadeh S. Mechanism of nanocapsules of matricaria recutita l. extract formation by the emulsion–diffusion process. Superlattices Microstruct 2011;50:340–9.

Esposito E, Cervellati F, Menegatti E, Nastruzzi C, Cortesi R. Spray dried eudragit microparticles as encapsulation devices for vitamin c. Int J Pharm 2002;242:329–34.

Essa S, Rabanel JM, Hildgen P. Effect of aqueous solubility of grafted moiety on the physicochemical properties of poly(d,l-lactide) (pla) based nanoparticles. Int J Pharm 2010;388:263–73.

Fàbregas A, Miñarro M, García-Montoya E, Pérez-Lozano P, Carrillo C et al. Impact of physical parameters on particle size and reaction yield when using the ionic gelation method to obtain cationic polymeric chitosan–tripolyphosphate nanoparticles. Int J Pharm 2013;446:199–204.

Falamarzian A, Lavasanifar A. Optimization of the hydrophobic domain in poly(ethylene oxide)-poly(ε-caprolactone) based nano-carriers for the solubilization and delivery of amphotericin b. Colloids Surf B Biointerfaces 2010;81:313–20.

Falco N, Reverchon E, Della Porta G. Injectable plga/hydrocortisone formulation produced by continuous supercritical emulsion extraction. Int J Pharm 2013;441:589–97.

Florindo HF, Pandit S, Lacerda L, Gonçalves LMD, Alpar HO, Almeida AJ. The enhancement of the immune response against s. equi antigens through the intranasal administration of poly-ε-caprolactone-based nanoparticles. Biomaterials 2009;30:879–91.

Fonseca C, Simões S, Gaspar R. Paclitaxel-loaded plga nanoparticles: preparation, physicochemical characterization and in vitro anti-tumoral activity. J Control Release 2002;83:273–86.

Fredriksen BN, Grip J. Plga/pla micro- and nanoparticle formulations serve as antigen depots and induce elevated humoral responses after immunization of atlantic salmon (salmo salar l.). Vaccine 2012;30:656–67.

Gaignaux A, Réeff J, Siepmann F, Siepmann J, De Vriese C et al. Development and evaluation of sustained-release clonidine-loaded plga microparticles. Int J Pharm 2012;437:20–8.

Galindo-Rodríguez SA, Puel F, Briançon S, Allémann E, Doelker E, Fessi H. Comparative scale-up of three methods for producing ibuprofen-loaded nanoparticles. Eur J Pharm Sci 2005;25:357–67.

Gan Q, Wang T, Cochrane C, McCarron P. Modulation of surface charge, particle size and morphological properties of chitosan–tpp nanoparticles intended for gene delivery. Colloids Surf B Biointerfaces 2005;44:65–73.

Gander B, Johansen P, Nam-Trân H, Merkle HP. Thermodynamic approach to protein microencapsulation into poly(d,l-lactide) by spray drying. Int J Pharm 1996;129:51–61.

Garekani HA, Moghaddam ZF, Sadeghi F. Organic solution versus aqueous dispersion of eudragit rs in preparation of sustained release microparticles of theophylline using spray drying. Colloids Surf B Biointerfaces 2013;108:374–9.

Gennes P-G. Scaling concepts in polymer physics. Ithaca, NY: Cornell University Press, 197.

Gèze A, Aous S, Baussanne I, Putaux J, Defaye J, Wouessidjewe D. Influence of chemical structure of amphiphilic β-cyclodextrins on their ability to form stable nanoparticles. Int J Pharm 2002;242:301–5.

Giri TK, Choudhary C, Ajazuddin, Alexander A, Badwaik H, Tripathi DK. Prospects of pharmaceuticals and biopharmaceuticals loaded microparticles prepared by double emulsion technique for controlled delivery. Saudi Pharm J 2013;21:125–41.

Giunchedi P, Juliano C, Gavini E, Cossu M, Sorrenti M. Formulation and in vivo evaluation of chlorhexidine buccal tablets prepared using drug-loaded chitosan microspheres. Eur J Pharm Biopharm 2002;53:233–9.

Gou M, Zheng L, Peng X, Men K, Zheng X et al. Poly(ε-caprolactone)–poly(ethylene glycol)–poly(ε-caprolactone) (pcl–peg–pcl) nanoparticles for honokiol delivery in vitro. Int J Pharm 2009;375:170–6.

Govender T, Stolnik S, Garnett MC, Illum L, Davis SS. Plga nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug. J Control Release 1999;57:171–85.

Govender T, Riley T, Ehtezazi T, Garnett MC, Stolnik S et al. Defining the drug incorporation properties of pla–peg nanoparticles. Int J Pharm 2000;199:95–110.

Guenet J-M. Thermoreversible gelation of polymers and biopolymers. New York: Academic Press, 1992.

Guerreiro LH, Da Silva D, Ricci-Junior E, Girard-Dias W, Mascarenhas CM et al. Polymeric particles for the controlled release of human amylin. Colloids Surf B Biointerfaces 2012;94:101–6.

Gupta AP, Kumar V. New emerging trends in synthetic biodegradable polymers – polylactide: a critique. Eur Polym J 2007;43:4053–74.

Gupta V, Ahsan F. Influence of pei as a core modifying agent on plga microspheres of pge_1, a pulmonary selective vasodilator. Int J Pharm 2011;413:51–62.

Gyulai G, Pénzes CB, Mohai M, Csempesz F, Kiss É. Influence of surface properties of polymeric nanoparticles on their membrane affinity. Eur Polym J 2013;49:2495-503.

Hachicha W, Kodjikian L, Fessi H. Preparation of vancomycin microparticles: importance of preparation parameters. Int J Pharm 2006;324:176–84.

Han S, Li M, Lin X, Gao H, Wu Y. Construction of amphiphilic copolymer nanoparticles based on gelatin as drug carriers for doxorubicin delivery. Colloids Surf B Biointerfaces 2013;102:833–41.

Hao S, Wang B, Wang Y, Zhu L, Wang B, Guo T. Preparation of eudragit l 100-55 enteric nanoparticles by a novel emulsion diffusion method. Colloids Surf B Biointerfaces 2013;108:127–33.

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

Haznedar S, Dortunç B. Preparation and in vitro evaluation of eudragit microspheres containing acetazolamide. Int J Pharm 2004;269:131–40.

He P, Davis SS, Illum L. Chitosan microspheres prepared by spray drying. Int J Pharm 1999;187:53–65.

He C, Yin L, Tang C, Yin C. Multifunctional polymeric nanoparticles for oral delivery of tnf-α sirna to macrophages. Biomaterials 2013;34:2843–54.

Hegazy N, Demirel M, Yazan Y. Preparation and in vitro evaluation of pyridostigmine bromide microparticles. Int J Pharm 2002;242:171–4.

Hernán Pérez de la Ossa D, Ligresti A, Gil-Alegre ME, Aberturas MR, Molpeceres J et al. Poly-ε-caprolactone microspheres as a drug delivery system for cannabinoid administration: development, characterization and in vitro evaluation of their antitumoral efficacy. J Control Release 2012;161:927–32.

Hombreiro Pérez M, Zinutti C, Lamprecht A, Ubrich N, Astier A et al. The preparation and evaluation of poly(ε-caprolactone) microparticles containing both a lipophilic and a hydrophilic drug. J Control Release 2000;65:429–38.

Hu Y, Jiang X, Ding Y, Zhang L, Yang C et al. Preparation and drug release behaviors of nimodipine-loaded poly(caprolactone)–poly(ethylene oxide)–polylactide amphiphilic copolymer nanoparticles. Biomaterials 2003;24:2395–404.

Huang Y-Y, Chung T-W, Tzeng T. Drug release from pla/peg microparticulates. Int J Pharm 1997;156:9–15.

Huang Y, Gao H, Gou M, Ye H, Liu Y et al. Acute toxicity and genotoxicity studies on poly(ε-caprolactone)-poly(ethylene glycol)-poly(ε-caprolactone) nanomaterials. Mutat Res Toxicol Environ Mutagen 2010;696:101–6.

Hyvönen S, Peltonen L, Karjalainen M, Hirvonen J. Effect of nanoprecipitation on the physicochemical properties of low molecular weight poly(l-lactic acid) nanoparticles loaded with salbutamol sulphate and beclomethasone dipropionate. Int J Pharm 2005;295:269–81.

Iler RK. Multilayers of colloidal particles. J Colloid Interface Sci 1966;21:569–94.

Illum L, Fisher AN, Jabbal-Gill I, Davis SS. Bioadhesive starch microspheres and absorption enhancing agents act synergistically to enhance the nasal absorption of polypeptides. Int J Pharm 2001;222:109–19.

Ishihara T, Takahashi M, Higaki M, Mizushima Y, Mizushima T. Preparation and characterization of a nanoparticulate formulation composed of peg-pla and pla as anti-inflammatory agents. Int J Pharm 2010;385:170–5.

Jafarinejad S, Gilani K, Moazeni E, Ghazi-Khansari M, Najafabadi AR, Mohajel N. Development of chitosan-based nanoparticles for pulmonary delivery of itraconazole as dry powder formulation. Powder Technol 2012;222:65–70.

Jain AK, Swarnakar NK, Godugu C, Singh RP, Jain S. The effect of the oral administration of polymeric nanoparticles on the efficacy and toxicity of tamoxifen. Biomaterials 2011;32:503–15.

Javadzadeh Y, Ahadi F, Davaran S, Mohammadi G, Sabzevari A, Adibkia K. Preparation and physicochemical characterization of naproxen-plga nanoparticles. Colloids Surf B Biointerfaces 2010;81:498–502.

Jordan F, Naylor A, Kelly CA, Howdle SM, Lewis A, Illum L. Sustained release hgh microsphere formulation produced by a novel supercritical fluid technology: in vivo studies. J Control Release 2010;141:153–60.

Kadam RS, Tyagi P, Edelhauser HF, Kompella UB. Influence of choroidal neovascularization and biodegradable polymeric particle size on transscleral sustained delivery of triamcinolone acetonide. Int J Pharm 2012;434:140–7.

Kalani M, Yunus R. Effect of supercritical fluid density on nanoencapsulated drug particle size using the supercritical antisolvent method. Int J Nanomed 2012;7:2165–72.

Kang Y, Wu J, Yin G, Huang Z, Yao Y et al. Preparation, characterization and in vitro cytotoxicity of indomethacin-loaded plla/plga microparticles using supercritical co2 technique. Eur J Pharm Biopharm 2008;70:85–97.

Kang Y, Yang C, Ouyang P, Yin G, Huang Z et al. The preparation of bsa-plla microparticles in a batch supercritical anti-solvent process. Carbohydr Polym 2009;77:244–9.

Kanthamneni N, Sharma S, Meenach SA, Billet B, Zhao J-C et al. Enhanced stability of horseradish peroxidase encapsulated in acetalated dextran microparticles stored outside cold chain conditions. Int J Pharm 2012;431:101–10.

Karataş A, Sonakin O, Kiliçarslan M, Baykara T. Poly (epsilon-caprolactone) microparticles containing levobunolol hcl prepared by a multiple emulsion (w/o/w) solvent evaporation technique: effects of some formulation parameters on microparticle characteristics. J Microencapsul 2009;26:63–74.

Kamchanajindanun J, Srisa-ard M, Baimark Y. Genipin-cross-linked chitosan microspheres prepared by a water-in-oil emulsion solvent diffusion method for protein delivery. Carbohydr Polym 2011;85:674–80.

Katara R, Majumdar DK. Eudragit rl 100-based nanoparticulate system of aceclofenac for ocular delivery. Colloids Surf B Biointerfaces 2013;103:455–62.

Katare YK, Muthukumaran T, Panda AK. Influence of particle size, antigen load, dose and additional adjuvant on the immune response from antigen loaded pla microparticles. Int J Pharm 2005;301:149–60.

Kauffman KJ, Kanthamneni N, Meenach SA, Pierson BC, Bachelder EM, Ainslie KM. Optimization of rapamycin-loaded acetalated dextran microparticles for immunosuppression. Int J Pharm 2012;422:356–63.

Keegan GM, Smart JD, Ingram MJ, Barnes L-M, Burnett GR, Rees GD. Chitosan microparticles for the controlled delivery of fluoride. J Dent 2012;40:229–40.

Khaled KA, Sarhan HA, Ibrahim MA, Ali AH, Naguib YW. Prednisolone-loaded plga microspheres. in vitro characterization and in vivo application in adjuvant-induced arthritis in mice. AAPS Pharm Sci Tech 2010;11:859–69.

Khalil NM, do Nascimento TCF, Casa DM, Dalmolin LF, de Mattos AC et al. Pharmacokinetics of curcumin-loaded plga and plga-peg blend nanoparticles after oral administration in rats. Colloids Surf B Biointerfaces 2013;101:353–60.

Khalil SKH, El-Feky GS, El-Banna ST, Khalil WA. Preparation and evaluation of warfarin-β-cyclodextrin loaded chitosan nanoparticles for transdermal delivery. Carbohydr Polym 2012;90:1244–53.

Khan MS, Vishakante GD, Bathool A. Development and characterization of brimonidine tartrate loaded eudragit nanosuspensions for ocular drug delivery. J Colloid Sci Biotechnol 2012;1:122–8.

Khayata N, Abdelwahed W, Chehna MF, Charcosset C, Fessi H. Preparation of vitamin e loaded nanocapsules by the nanoprecipitation method: from laboratory scale to large scale using a membrane contactor. Int J Pharm 2012;423:419–27.

Khoee S, Yaghoobian M. An investigation into the role of surfactants in controlling particle size of polymeric nanocapsules containing penicillin-g in double emulsion. Eur J Med Chem 2009;44:2392–9.

Khoee S, Sattari A, Atyabi F. Physico-chemical properties investigation of cisplatin loaded polybutyladipate (pba) nanoparticles prepared by w/o/w. Mater Sci Eng C 2012;32:1078–86.

Konecsni K, Low NH, Nickerson MT. Chitosan–tripolyphosphate submicron particles as the carrier of entrapped rutin. Food Chem 2012;134:1775–9.

Koten JW, Van Luyn MJA, Cadée JA, Brouwer L, Hennink WE et al. Il-2 loaded dextran microspheres with attractive histocompatibility properties for local il-2 cancer therapy. Cytokine 2003;24:57–66.

Krishnakumar N, Sulfikkarali N, Rajendra Prasad N, Karthikeyan S. Enhanced anticancer activity of naringenin-loaded nanoparticles in human cervical (hela) cancer cells. Biomed Prev Nutr 2011;1:223–31.

Kumar A, Gupta GK, Khedgikar V, Gautam J, Kushwaha P et al. In vivo efficacy studies of layer-by-layer nano-matrix bearing kaempferol for the conditions of osteoporosis: a study in ovariectomized rat model. Eur J Pharm Biopharm 2012;82:508–17.

Kumari A, Yadav SK, Pakade YB, Kumar V, Singh B et al. Nanoencapsulation and characterization of albizia chinensis isolated antioxidant quercitrin on pla nanoparticles. Colloids Surf B Biointerfaces 2011;82:224–32.

Kwon H-Y, Lee J-Y, Choi S-W, Jang Y, Kim J-H. Preparation of plga nanoparticles containing estrogen by emulsification–diffusion method. Colloids Surf Physicochem Eng Asp 2001;182:123–30.

Labruère R, Sicard R, Cormier R, Turos E, West L. Poly(vinyl benzoate) nanoparticles for molecular delivery: studies on their preparation and in vitro properties. J Control Release 2010;148:234–40.

Lamalle-Bernard D, Munier S, Compagnon C, Charles M-H, Kalyanaraman VS et al. Coadsorption of hiv-1 p24 and gp120 proteins to surfactant-free anionic pla nanoparticles preserves antigenicity and immunogenicity. J Control Release 2006;115:57–67.

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

Le Ray A-M, Chiffoleau S, Iooss P, Grimandi G, Gouyette A et al. Vancomycin encapsulation in biodegradable poly(ε-caprolactone) microparticles for bone implantation. influence of the formulation process on size, drug loading, in vitro release and cytocompatibility. Biomaterials 2003;24:443–9.

Lee SJ, Hong G-Y, Jeong Y-I, Kang M-S, Oh J-S et al. Paclitaxel-incorporated nanoparticles of hydrophobized polysaccharide and their antitumor activity. Int J Pharm 2012;433:121–8.

Lemos-Senna E, Wouessidjewe D, Duchêne D, Lesieur S. Amphiphilic cyclodextrin nanospheres: particle solubilization and reconstitution by the action of a non-ionic detergent. Colloids Surf B Biointerfaces 1998;10:291–301.

Leo E, Forni F, Bernabei MT. Surface drug removal from ibuprofen-loaded pla microspheres. Int J Pharm 2000;196:1–9.

Li B, Wang Q, Wang X, Wang C, Jiang X. Preparation, drug release and cellular uptake of doxorubicin-loaded dextran-b-poly(ε-caprolactone) nanoparticles. Carbohydr Polym 2013;93:430–7.

Li P, Wang Y, Peng Z, She F, Kong L. Development of chitosan nanoparticles as drug delivery systems for 5-fluorouracil and leucovorin blends. Carbohydr Polym 2011;85:698–704.

Li X, Chang S, Du G, Li Y, Gong J et al. Encapsulation of azithromycin into polymeric microspheres by reduced pressure-solvent evaporation method. Int J Pharm 2012;433:79–88.

Liang D, Fu X, Liao M, Yuan W, Su J. Development of dextran microparticles loaded with il-1ra of high-encapsulation efficiency and high-bioactivity by a novel method without exposing il-1ra to water–oil interfaces. Powder Technol 2013;235:299–302.

Limayem Blouza I, Charcosset C, Sfar S, Fessi H. Preparation and characterization of spironolactone-loaded nanocapsules for paediatric use. Int J Pharm 2006;325:124–31.

Lince F, Marchisio DL, Barresi AA. Strategies to control the particle size distribution of poly-ε-caprolactone nanoparticles for pharmaceutical applications. J Colloid Interface Sci 2008;322:505–15.

Ma J, Feng P, Ye C, Wang Y, Fan Y. An improved interfacial coacervation technique to fabricate biodegradable nanocapsules of an aqueous peptide solution from polylactide and its block copolymers with poly(ethylene glycol). Colloid Polym Sci 2001;279:387–92.

Mahapatro A, Singh DK. Biodegradable nanoparticles are excellent vehicle for site directed in-vivo delivery of drugs and vaccines. J Nanobiotechnol 2011;9:55.

Manchanda R, Fernandez-Fernandez A, Nagesetti A, McGoron AJ. Preparation and characterization of a polymeric (plga) nanoparticulate drug delivery system with simultaneous incorporation of chemotherapeutic and thermo-optical agents. Colloids Surf B Biointerfaces 2010;75:260–7.

Mao S, Sun W, Kissel T. Chitosan-based formulations for delivery of dna and sirna. Adv Drug Deliv Rev 2010;62:12–27.

Martin TM, Bandi N, Shulz R, Roberts CB, Kompella UB. Preparation of budesonide and budesonide-pla microparticles using supercritical fluid precipitation technology. AAPS Pharm Sci Tech 2002;3:E18.

Martín-Banderas L, Alvarez-Fuentes J, Durán-Lobato M, Prados J, Melguizo C, Fernández-Arévalo M et al. Cannabinoid derivate-loaded plga nanocarriers for oral administration: formulation, characterization, and cytotoxicity studies. Int J Nanomed 2012;7:5793–806.

Masson V, Maurin F, Fessi H, Devissaguet JP. Influence of sterilization processes on poly(ε-caprolactone) nanospheres. Biomaterials 1997;18:327–35.

Maya S, Kumar LG, Sarmento B, Sanoj Rejinold N, Menon D et al. Cetuximab conjugated o-carboxymethyl chitosan nanoparticles for targeting egfr overexpressing cancer cells. Carbohydr Polym 2013;93:661–9.

Mazzaferro S, Bouchemal K, Maksimenko A, Skanji R, Opolon P, Ponchel G. Reduced intestinal toxicity of docetaxel loaded into mucoadhesive nanoparticles, in mouse xenograft model. J Colloid Sci Biotechnol 2012;1:210–7.

Mazzarino L, Travelet C, Ortega-Murillo S, Otsuka I, Pignot-Paintrand I et al. Elaboration of chitosan-coated nanoparticles loaded with curcumin for mucoadhesive applications. J Colloid Interface Sci 2012;370:58–66.

Mehvar R. Dextrans for targeted and sustained delivery of therapeutic and imaging agents. J Control Release 2000;69:1–25.

Memişoğlu E, Bochot A, Şen M, Duchêne D, Hıncal AA. Non-surfactant nanospheres of progesterone inclusion complexes with amphiphilic β-cyclodextrins. Int J Pharm 2003;251:143–53.

Menei P, Montero-Menei C, Venier M-C, Benoit J-P. Drug delivery into the brain using poly(lactide-co-glycolide) microspheres. Expert Opin Drug Deliv 2005;2:363–76.

Miladi K, Sfar S, Fessi H, Elaissari A. Drug carriers in osteoporosis: preparation, drug encapsulation and applications. Int J Pharm 2013;445:181–95.

Minost A, Delaveau J, Bolzinger M-A, Fessi H, Elaissari A. Nanoparticles via nanoprecipitation process. Recent Pat Drug Deliv Formul 2012;6:250–8.

Miyazaki Y, Onuki Y, Yakou S, Takayama K. Effect of temperature-increase rate on drug release characteristics of dextran microspheres prepared by emulsion solvent evaporation process. Int J Pharm 2006;324:144–51.

Mu L, Hou L, Guo D, Xiao X, Mao P, Yang X. Preparation and characterization of teniposide plga nanoparticles and their uptake in human glioblastoma u87mg cells. Int J Pharm 2012;436:815–24.

Mohajel N, Najafabadi AR, Azadmanesh K, Vatanara A, Moazeni E et al. Optimization of a spray drying process to prepare dry powder microparticles containing plasmid nanocomplex. Int J Pharm 2012; 423:577–85.

Mora-Huertas CE, Fessi H, Elaissari A. Polymer-based nanocapsules for drug delivery. Int J Pharm 2010;385:113–42.

Mora-Huertas CE, Garrigues O, Fessi H, Elaissari A. Nanocapsules prepared via nanoprecipitation and emulsification-diffusion methods: comparative study. Eur J Pharm Biopharm 2012;80:235–9.

Morales-Cruz M, Flores-Fernández GM, Morales-Cruz M, Orellano EA, Rodriguez-Martinez JA et al. Two-step nanoprecipitation for the production of protein-loaded plga nanospheres. Results Pharma Sci 2012;2:79–85.

Mu L, Feng S. Fabrication, characterization and in vitro release of paclitaxel (taxol®) loaded poly (lactic-co-glycolic acid) microspheres prepared by spray drying technique with lipid/cholesterol emulsifiers. J Control Release 2001;76:239–54.

Mutil P, Kaur J, Kumar K, Yadav AB, Sharma R, Misra A. Inhalable microparticles containing large payload of anti-tuberculosis drugs. Eur J Pharm Sci 2007;32:140–50.

Nahar M, Mishra D, Dubey V, Jain NK. Development, characterization, and toxicity evaluation of amphotericin b–loaded gelatin nanoparticles. Nanomed Nanotechnol Biol Med 2008;4:252–61.

Nchilla B, Bergkvist M, Popat K, Desai T. Purified and surfactant-free coenzyme q10-loaded biodegradable nanoparticles. Int J Pharm 2008;348:107–14.

O'Donnell PB, McGinity JW. Preparation of microspheres by the solvent evaporation technique. Adv Drug Deliv Rev 1997;28:25–42.

Osman R, Kan PL, Awad G, Mortada N, El-Shamy A-E, Alpar O. Enhanced properties of discrete pulmonary deoxyribonuclease i (dnasei) loaded plga nanoparticles during encapsulation and activity determination. Int J Pharm 2011;408:257–65.

Palazzo F, Giovagnoli S, Schoubben A, Blasi P, Rossi C, Ricci M. Development of a spray-drying method for the formulation of respirable microparticles containing ofloxacin-palladium complex. Int J Pharm 2013;440:273–82.

Pariot N, Edwards-Lévy F, Andry M-C, Lévy M-C. Cross-linked β-cyclodextrin microcapsules. ii. retarding effect on drug release through semi-permeable membranes. Int J Pharm 2002;232:175–81.

Park JH, Saravanakumar G, Kim K, Kwon IC. Targeted delivery of low molecular drugs using chitosan and its derivatives. Adv Drug Deliv Rev 2010;62: 28–41.

Patil SB, Sawant KK. Chitosan microspheres as a delivery system for nasal insufflation. Colloids Surf B Biointerfaces 2011;84:384–9.

Péan JM, Venier-Julienne MC, Boury F, Menei P, Denizot B, Benoit JP. Ngf release from poly(d,l-lactide-co-glycolide) microspheres. effect of some formulation parameters on encapsulated ngf stability. J Control Release 1998;56:175–87.

Pignatello R, Bucolo C, Ferrara P, Maltese A, Puleo A, Puglisi G. Eudragit rs100® nanosuspensions for the ophthalmic controlled delivery of ibuprofen. Eur J Pharm Sci 2002;16:53–61.

Pillai O, Panchagnula R. Polymers in drug delivery. Curr Opin Chem Biol 2001;5:447–51.

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

Pinto Reis C, Neufeld RJ, Ribeiro AJ, Veiga F. Nanoencapsulation i. methods for preparation of drug-loaded polymeric nanoparticles. Nanomed Nanotechnol Biol Med 2006;2:8–21.

Poletto FS, Fiel LA, Donida B, Ré MI, Guterres SS, Pohlmann AR. Controlling the size of poly(hydroxybutyrate-co-hydroxyvalerate) nanoparticles prepared by emulsification–diffusion technique using ethanol as surface agent. Colloids Surf Physicochem Eng Asp 2008;324:105–12.

Powell TJ, Palath N, DeRome ME, Tang J, Jacobs A, Boyd JG. Synthetic nanoparticle vaccines produced by layer-by-layer assembly of artificial biofilms induce potent protective t-cell and antibody responses in vivo. Vaccine 2011;29:558–69.

Prata AS, Garcia L, Tonon RV, Hubinger MD. Wall material selection for encapsulation by spray drying. J Colloid Sci Biotechnol 2013;2:86–92.

Prior S, Gamazo C, Irache J, Merkle H, Gander B. Gentamicin encapsulation in pla/plga microspheres in view of treating brucella infections. Int J Pharm 2000;196:115–25.

Qi W, Yan X, Fei J, Wang A, Cui Y, Li J. Triggered release of insulin from glucose-sensitive enzyme multilayer shells. Biomaterials 2009;30:2799–806.

Quintanar-Guerrero D, Fessi H, Allémann E, Doelker E. Influence of stabilizing agents and preparative variables on the formation of poly(d,l-lactic acid) nanoparticles by an emulsification-diffusion technique. Int J Pharm 1996;143:133–41.

Radtchenko IL, Sukhorukov GB, Möhwald H. Incorporation of macromolecules into polyelectrolyte micro- and nanocapsules via surface controlled precipitation on colloidal particles. Colloids Surf Physicochem Eng Asp 2002;202:127–33.

Raffin RP, Colomé LM, Schapoval EES, Pohlmann AR, Guterres SS. Increasing sodium pantoprazole photostability by microencapsulation: effect of the polymer and the preparation technique. Eur J Pharm Biopharm 2008;69:1014–8.

Rao JP, Geckeler KE. Polymer nanoparticles: preparation techniques and size-control parameters. Prog Polym Sci 2011;36:887–913.

Rattes ALR, Oliveira WP. Spray drying conditions and encapsulating composition effects on formation and properties of sodium diclofenac microparticles. Powder Technol 2007;171:7–14.

Raval JP, Naik DR, Amin KA, Patel PS. Controlled-release and antibacterial studies of doxycycline-loaded poly(ε-caprolactone) microspheres. J Saudi Chem Soc, 2011, epub ahead of print, http://dx.doi.org/10.1016/j.jscs.2011.11.004

Rawat A, Burgess DJ. Effect of ethanol as a processing co-solvent on the plga microsphere characteristics. Int J Pharm 2010;394:99–105.

Rees DA, Welsh EJ. Secondary and tertiary structure of polysaccharides in solutions and gels. Angew Chem Int Ed Engl 1977;16:214–24.

Rosset V, Ahmed N, Zaanoun I, Stella B, Fessi H, Elaissari A. Elaboration of argan oil nanocapsules containing naproxen for cosmetic and transdermal local application. J Colloid Sci Biotechnol 2012;1: 218–24.

Ruan G, Feng S-S. Preparation and characterization of poly(lactic acid)–poly(ethylene glycol)–poly(lactic acid) (pla–peg–pla) microspheres for controlled release of paclitaxel. Biomaterials 2003;24:5037–44.

Sacchetin PSC, Morales AR, Moraes ÂM, Rosa P de TV e. Formation of pla particles incorporating 17α-methyltestosterone by supercritical fluid technology. J Supercrit Fluids 2013;77:52–62.

Sajadi Tabassi SA, Tafaghodi M, Jaafari MR. Induction of high antitoxin titers against tetanus toxoid in rabbits by intranasal immunization with dextran microspheres. Int J Pharm 2008;360:12–7.

Sane A, Limtrakul J. Formation of retinyl palmitate-loaded poly(l-lactide) nanoparticles using rapid expansion of supercritical solutions into liquid solvents (resolv). J Supercrit Fluids 2009;51:230–7.

Sanli D, Bozbag SE, Erkey C. Synthesis of nanostructured materials using supercritical co2: part i. physical transformations. J Mater Sci 2012;47: 2995–3025.

Santos DT, Albarelli JQ, Beppu MM, Meireles MAA. Stabilization of anthocyanin extract from jabuticaba skins by encapsulation using supercritical co2 as solvent. Food Res Int 2013;50:617–24.

Sastre RL, Olmo R, Teijón C, Muñíz E, Teijón JM, Blanco MD. 5-fluorouracil plasma levels and biodegradation of subcutaneously injected drug-loaded microspheres prepared by spray-drying poly(d,l-lactide) and poly(d,l-lactide-co-glycolide) polymers. Int J Pharm 2007;338:180–90.

Seju U, Kumar A, Sawant KK. Development and evaluation of olanzapine-loaded plga nanoparticles for nose-to-brain delivery: in vitro and in vivo studies. Acta Biomater 2011;7:4169–76.

Seo D-H, Jeong Y-I, Kim D-G, Jang M-J, Jang M-K, Nah J-W. Methotrexate-incorporated polymeric nanoparticles of methoxy poly(ethylene glycol)-grafted chitosan. Colloids Surf B Biointerfaces 2009;69:157–63.

Seremeta KP, Chiappetta DA, Sosnik A. Poly(ε-caprolactone), eudragit® rs 100 and poly(ε-caprolactone)/eudragit® rs 100 blend submicron particles for the sustained release of the antiretroviral efavirenz. Colloids Surf B Biointerfaces 2013;102:441–9.

Shao Y, Zhu B, Li J, Liu X, Tan X, Yang X. Novel chitosan microsphere-templated microcapsules suitable for spontaneous loading of heparin. Mater Sci Eng C 2009;29:936–41.

Silva AL, Rosalia RA, Sazak A, Carstens MG, Ossendorp F et al. Optimization of encapsulation of a synthetic long peptide in plga nanoparticles: low-burst release is crucial for efficient cd8(+) t cell activation. Eur J Pharm Biopharm 2013;83:338–45.

Simşek S, Eroğlu H, Kurum B, Ulubayram K. Brain targeting of atorvastatin loaded amphiphilic plga-b-peg nanoparticles. J Microencapsul 2013;30:10–20.

Singh M, Kazzaz J, Ugozzoli M, Chesko J, O'Hagan DT. Charged polylactide co-glycolide microparticles as antigen delivery systems. Expert Opin Biol Ther 2004;4:483–91.

Sinha VR, Bansal K, Kaushik R, Kumria R, Trehan A. Poly-epsilon-caprolactone microspheres and nanospheres: an overview. Int J Pharm 2004;278:1–23.

Siqueira-Moura MP, Primo FL, Espreafico EM, Tedesco AC. Development, characterization, and photocytotoxicity assessment on human melanoma of chloroaluminum phthalocyanine nanocapsules. Mater Sci Eng C 2013;33:1744–52.

Soares ASP. A special issue on applications of microencapsulation. J Colloid Sci Biotechnol 2013;2:77.

Sollohub K, Cal K. Spray drying technique: ii. current applications in pharmaceutical technology. J Pharm Sci 2010;99:587–97.

Song KC, Lee HS, Choung IY, Cho KI, Ahn Y, Choi EJ. The effect of type of organic phase solvents on the particle size of poly(d,l-lactide-co-glycolide) nanoparticles. Colloids Surf Physicochem Eng Asp 2006;276:162–7.

Song X, Zhao Y, Wu W, Bi Y, Cai Z et al. Plga nanoparticles simultaneously loaded with vincristine sulfate and verapamil hydrochloride: systematic study of particle size and drug entrapment efficiency. Int J Pharm 2008;350:320–9.

Sou T, Meeusen EN, de Veer M, Morton DAV, Kaminskas LM, McIntosh MP. New developments in dry powder pulmonary vaccine delivery. Trends Biotechnol 2011;29:191–8.

Souguir H, Salaün F, Douillet P, Vroman I, Chatterjee S. Nanoencapsulation of curcumin in polyurethane and polyurea shells by an emulsion diffusion method Chem Eng J 2013;221:133–45.

Stulzer HK, Tagliari MP, Parize AL, Silva MAS, Laranjeira MCM. Evaluation of cross-linked chitosan microparticles containing acyclovir obtained by spray-drying. Mater Sci Eng C 2009;29:387–92.

Su Z, Sun F, Shi Y, Jiang C, Meng Q et al. Effects of formulation parameters on encapsulation efficiency and release behavior of risperidone poly(d,l-lactide-co-glycolide) microsphere. Chem Pharm Bull (Tokyo) 2009;57:1251–6.

Sukhorukov GB, Donath E, Lichtenfeld H, Knippel E, Knippel M et al. Layer-by-layer self assembly of polyelectrolytes on colloidal particles. Colloids Surf Physicochem Eng Asp 1998;137:253–66.

Suksiriworapong J, Sripha K, Kreuter J, Junyaprasert VB. Functionalized (poly(ε-caprolactone))2-poly(ethyleneglycol) nanoparticles with grafting nicotinic acid as drug carriers. Int J Pharm 2012;423:562–70.

Surassmo S, Min S-G, Bejrapha P, Choi M-J. Effects of surfactants on the physical properties of capsicum oleoresin-loaded nanocapsules formulated through the emulsion–diffusion method. Food Res Int 2010;43:8–17.

Tawfeek HM. Evaluation of peg and mpeg-co-(pga-co-pdl) microparticles loaded with sodium diclofenac. Saudi Pharm J 2013;21:387–97.

Tewa-Tagne P, Briançon S, Fessi H. Spray-dried microparticles containing polymeric nanocapsules: formulation aspects, liquid phase interactions and particles characteristics. Int J Pharm 2006;325:63–74.

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

Thomas C, Gupta V, Ahsan F. Influence of surface charge of plga particles of recombinant hepatitis b surface antigen in enhancing systemic and mucosal immune responses. Int J Pharm 2009;379:41–50.

Trapani A, Laquintana V, Denora N, Lopedota A, Cutrignelli A et al. Eudragit rs 100 microparticles containing 2-hydroxypropyl-β-cyclodextrin and glutathione: physicochemical characterization, drug release and transport studies. Eur J Pharm Sci 2007;30:64–74.

Tse MT, Blatchford C, Oya Alpar H. Evaluation of different buffers on plasmid dna encapsulation into plga microparticles. Int J Pharm 2009;370:33–40.

Valot P, Baba M, Nedelec J-M, Sintes-Zydowicz N. Effects of process parameters on the properties of biocompatible ibuprofen-loaded microcapsules. Int J Pharm 2009;369:53–63.

Van de Ven H, Vermeersch M, Matheeussen A, Vandervoort J, Weyenberg W et al. Plga nanoparticles loaded with the antileishmanial saponin β-aescin: factor influence study and in vitro efficacy evaluation. Int J Pharm 2011;420:122–32.

Van de Ven H, Paulussen C, Feijens PB, Matheeussen A, Rombaut P et al. Plga nanoparticles and nanosuspensions with amphotericin b: potent in vitro and in vivo alternatives to fungizone and ambisome. J Control Release 2012;161:795–803.

Van der Graaf S, Schroën CGPH, Boom RM. Preparation of double emulsions by membrane emulsification—a review. J Membr Sci 2005;251:7–15.

Vanderhoff JW, El-Aasser MS, Ugelstad J. Polymer emulsification process. U.S. Patent 4,177,177, December 1979.

Vandervoort J, Ludwig A. Preparation and evaluation of drug-loaded gelatin nanoparticles for topical ophthalmic use. Eur J Pharm Biopharm 2004;57:251–61.

Vauthier C, Bouchemal K. Methods for the preparation and manufacture of polymeric nanoparticles. Pharm Res 2009;26:1025–58.

Vergaro V, Scarlino F, Bellomo C, Rinaldi R, Vergara D et al. Drug-loaded polyelectrolyte microcapsules for sustained targeting of cancer cells. Adv Drug Deliv Rev 2011;63:847–64.

Vila A, Gill H, McCallion O, Alonso MJ. Transport of pla-peg particles across the nasal mucosa: effect of particle size and peg coating density. J Control Release 2004;98:231–44.

Vila A, Sánchez A, Évora C, Soriano I, McCallion O, Alonso MJ. Pla-peg particles as nasal protein carriers: the influence of the particle size. Int J Pharm 2005;292:43–52.

Vladisavljević GT, Williams RA. Recent developments in manufacturing particulate products from double-emulsion templates using membrane and microfluidic devices. In: Aserin A (ed.): Multiple emulsions: technology and applications (pp 121-64). New York: Wiley, 2008.

Wang F-J, Wang C-H. Sustained release of etanidazole from spray dried microspheres prepared by non-halogenated solvents. J Control Release 2002;81:263–80.

Wang G, Yu B, Wu Y, Huang B, Yuan Y, Liu CS. Controlled preparation and antitumor efficacy of vitamin e tpgs-functionalized plga nanoparticles for delivery of paclitaxel. Int J Pharm 2013;446:24–33.

Wang S, Guo S, Cheng L. Disodium norcantharidate loaded poly(ε-caprolactone) microspheres: i. preparation and evaluation. Int J Pharm 2008;350:130–7.

Xie H, Smith JW. Fabrication of plga nanoparticles with a fluidic nanoprecipitation system. J Nanobiotechnol 2010;8:18.

Xin H, Chen L, Gu J, Ren X, Wei Z, et al. Enhanced anti-glioblastoma efficacy by ptx-loaded pegylated poly(ε-caprolactone) nanoparticles: in vitro and in vivo evaluation. Int J Pharm 2010;402:238–47.

Yadav KS, Sawant KK. Formulation optimization of etoposide loaded plga nanoparticles by double factorial design and their evaluation. Curr Drug Deliv 2010;7:51–64.

Yan S, Zhu J, Wang Z, Yin J, Zheng Y, Chen X. Layer-by-layer assembly of poly(l-glutamic acid)/chitosan microcapsules for high loading and sustained release of 5-fluorouracil. Eur J Pharm Biopharm 2011;78:336–45.

Yang J, Park S-B, Yoon H-G, Huh Y-M, Haam S. Preparation of poly ε-caprolactone nanoparticles containing magnetite for magnetic drug carrier. Int J Pharm 2006;324:185–90.

Yang Z, Birkenhauer P, Julmy F, Chickering D, Ranieri JP et al. Sustained release of heparin from polymeric particles for inhibition of human vascular smooth muscle cell proliferation. J Control Release 1999;60:269–77.

EXCLI Journal 2014;13:28-57 – ISSN 1611-2156
Received: November 05, 2013, accepted: January 25, 2014, published: February 03, 2014

Yildiz A, John E, Özsoy Y, Araman A, Birchall JC et al. Inhaled extended-release microparticles of heparin elicit improved pulmonary pharmacodynamics against antigen-mediated airway hyper-reactivity and inflammation. J Control Release 2012;162:456–63.

Yoncheva K, Gómez S, Campanero MA, Gamazo C, Irache JM. Bioadhesive properties of pegylated nanoparticles. Expert Opin Drug Deliv 2005;2:205–18.

Yoo M-K, Kang S-K, Choi J-H, Park I-K, Na H-S et al. Targeted delivery of chitosan nanoparticles to peyer's patch using m cell-homing peptide selected by phage display techniquc. Biomaterials 2010;31: 7738–47.

Yordanov G. Influence of the preparation method on the physicochemical properties of econazole-loaded poly(butyl cyanoacrylate) colloidal nanoparticles. Colloids Surf Physicochem Eng Asp 2012;413:260–5.

Youm I, Murowchick JB, Youan B-BC. Entrapment and release kinetics of furosemide from pegylated nanocarriers. Colloids Surf B Biointerfaces 2012;94: 133–42.

Young S, Wong M, Tabata Y, Mikos AG. Gelatin as a delivery vehicle for the controlled release of bioactive molecules. J Control Release 2005;109:256–74.

Zakeri-Milani P, Loveymi BD, Jelvehgari M, Valizadeh H. The characteristics and improved intestinal permeability of vancomycin plga-nanoparticles as colloidal drug delivery system. Colloids Surf B Biointerfaces 2013;103:174–81.

Zambaux MF, Bonneaux F, Gref R, Maincent P, Dellacherie E et al. Influence of experimental parameters on the characteristics of poly(lactic acid) nanoparticles prepared by a double emulsion method. J Control Release 1998;50:31–40.

Zandanel C, Vauthier C. Poly (isobutylcyanoacrylate) nanoparticles decorated with chitosan: effect of conformation of chitosan chains at the surface on complement activation properties. J Colloid Sci Biotechnol 2012;1:68–81.

Zhang C, Li G, Wang Y, Cui F, Zhang J, Huang Q. Preparation and characterization of 5-fluorouracil-loaded plla-peg/peg nanoparticles by a novel supercritical co2 technique. Int J Pharm 2012;436:272–81.

Zhang F, Wu Q, Chen Z-C, Zhang M, Lin X-F. Hepatic-targeting microcapsules construction by self-assembly of bioactive galactose-branched polyelectrolyte for controlled drug release system. J Colloid Interface Sci 2008a;317:477–84.

Zhang F, Wu Q, Liu L-J, Chen Z-C, Lin X-F. Thermal treatment of galactose-branched polyelectrolyte microcapsules to improve drug delivery with reserved targetability. Int J Pharm 2008b;357:22–31.

Zhang H, Cui W, Bei J, Wang S. Preparation of poly(lactide-co-glycolide-co-caprolactone) nanoparticles and their degradation behaviour in aqueous solution. Polym Degrad Stab 2006;91:1929–36.

Zhao H, Gagnon J, Hafeli UO. Process and formulation variables in the preparation of injectable and biodegradable magnetic microspheres. Biomagn Res Technol 2007;5:2.

Zheng X, Kan B, Gou M, Fu S, Zhang J et al. Preparation of mpeg–pla nanoparticle for honokiol delivery in vitro. Int J Pharm 2010;386:262–7.

Zhou YZ, Alany RG, Chuang V, Wen J. Optimization of plga nanoparticles formulation containing l-dopa by applying the central composite design. Drug Dev Ind Pharm 2013;39:321–30.

La nanoprécipitation : de la préparation des particules aux applications *in vivo*

***Accepté** pour publication en tant que chapitre du livre*
« Polymer nanoparticles: A guide for design, preparation and development as nanomedecine »
***Editeurs :** Christine Vauthier et Gilles Ponchel*
***Maison d'édition :** Springer*

Cf. annexes

Les applications de l'encapsulation en ostéoporose

Publiée *sous forme d'une revue dans*
«International Journal of Pharmaceutics »
International Journal of Pharmaceutics *2013; 445: 181-195.*

La revue présente un état de l'art de l'encapsulation de molécules actives qui sont indiquées en ostéoporose. Tout d'abord, la problématique du traitement pharmacologique de l'ostéoporose est présentée. Par la suite, une explication des avantages qui peuvent être apportés par l'encapsulation des substances actives est exposée. Cette revue inclut aussi une description des différentes méthodes qui ont été utilisées pour l'encapsulation des molécules actives indiquées en ostéoporose. Les propriétés des particules obtenues sont aussi décrites. Les études *in vitro* et *in vivo* qui sont réalisées sur les particules préparées sont aussi discutées.

Les formes particulaires ont été largement développées pour améliorer l'efficacité des substances actives. En effet, l'encapsulation a permis d'améliorer la stabilité des substances actives, le ciblage et la prolongation de l'effet pharmacologique par la libération prolongée. En effet, l'intérêt qui a été prêté a l'encapsulation et les avantages qui ont été déjà obtenus grâce à cette approche, ont donné naissance à plusieurs applications en ostéoporose. Ainsi, le but de cette revue bibliographique est de rapporter toutes les formes particulaires qui ont été développées pour le traitement de l'ostéoporose. Tout d'abord, la problématique liée au traitement pharmacologique de l'ostéoporose a été présentée. En effet, de nombreuses molécules actives sont indiquées pour le traitement de l'ostéoporose. Cependant, il s'avère que la plupart de ces molécules présentent des problèmes de biodisponibilité suite à l'utilisation clinique. De telles limites correspondent à une faible solubilité, une mauvaise absorption ou un premier passage hépatique important. Pour les bisphosphonates, par exemple, l'absorption orale va de 0,7% (pour l'alendronate et le risédronate) jusqu'à uniquement 6% (pour l'étidronate et le tiludronate). Les œstrogènes et la progestérone subissent un premier passage hépatique important ce qui diminue fortement leur biodisponibilité. Le raloxifène, qui est un modulateur sélectif du récepteur à estrogènes, présente une faible biodisponibilité (< 2%) qui est due à une faible solubilité dans les liquides biologiques et à un effet de premier passage métabolique très important. La calcitonine est de nature protéique ce qui implique sa dégradation lorsqu'elle est administrée par la voie orale. En plus, sa demi-vie par voie parentérale est très courte (15-20 min). D'autres problèmes de tolérance peuvent s'ajouter surtout dans le cas des bisphosphonates avec des effets indésirables d'irritation au niveau œsophagien ou gastro-intestinal. Dans cette étude, nous nous sommes intéressés à la description de tous les véhicules qui ont été préparées pour une

utilisation en ostéoporose. Les techniques de préparation ainsi que les propriétés des particules obtenues ont été relevées et discutées. Les propriétés des molécules encapsulées et les différentes méthodes utilisées pour l'étude de leur efficacité *in vitro* et *in vivo* ont été aussi décrites. Un état de l'art de la recherche dans le domaine de l'encapsulation des molécules indiquées en ostéoporose est ainsi examiné. Les principes des différentes techniques d'encapsulation utilisées et les paramètres expérimentaux clés à maitriser ont été étudiés. Nous concluons que l'ostéoporose représente un problème majeur vu qu'elle est à l'origine de taux de morbidité et de mortalité élevés. L'approche d'encapsulation a été utilisée pour pallier les problématiques de biodisponibilité et de tolérance. Les formes préparées sont essentiellement des particules et des liposomes. Les techniques qui ont été les plus utilisées sont : l'émulsion évaporation du solvant, le spray-drying, la polymérisation en émulsion, la gélification ionique, l'hydratation du film lipidique, l'émulsion diffusion du solvant et l'adsorption des molécules actives sur des particules. Plusieurs types de polymères ont été utilisés avec une prédominance des polyesters biodégradables et des Eudragit®. Certaines approches se sont basées sur l'utilisation de polymères mucoadhésifs comme le chitosane et ses dérivés. D'autres méthodes reposent sur l'utilisation de polymères qui permettent une libération prolongée dans le but de minimiser le nombre de prises quotidiennes et améliorer, par conséquent, le confort du patient. La protection des molécules de nature protéique (contre l'action des enzymes) par l'intermédiaire de l'encapsulation a été aussi utilisée comme alternative. La plupart des vecteurs ont été préparés par la technique d'évaporation du solvant d'émulsion en utilisant principalement l'acide poly(lactique-co-glycolique) en tant que polymère. Presque toutes les voies d'administration ont été ciblées.

Beaucoup de paramètres expérimentaux ont influé le pourcentage d'encapsulation des molécules actives et leurs profils de libération *in vitro*. Par exemple, la nature du polymère est un paramètre clé qui a une influence sur les propriétés des particules préparées.

Les essais *in vitro* ont été réalisés en utilisant les cellules Caco-2. D'autres tests utilisaient des modèles cellulaires différents comme les cellules RAW264,7. Certaines études se sont basées sur l'essai de dissolution décrit par la pharmacopée. Par contre, les essais *in vivo* ont été réalisés principalement chez le rat. Certaines études procédaient à un marquage des particules préparées pour pouvoir étudier par la suite leur dépôt au niveau de l'os. D'autres études ont recherché de potentiels effets indésirables d'irritation au niveau de certains tissus (estomac, poumons). Des études pharmacocinétiques ont été aussi réalisées pour déterminer les concentrations plasmatiques, le profil de libération (immédiat ou prolongé) et la

biodisponibilité. D'autres études à caractère pharmacodynamique ont été aussi menées comme la détermination de la masse osseuse totale chez le rat après l'administration des particules.

Les avantages conférés par l'encapsulation ont été mis en évidence *in vitro* et *in vivo*. Par exemple, Mondal *et al.* (2012) ont démontré une augmentation de l'adhésion cellulaire des particules qu'ils ont préparées. Nasr *et al.* (2011) ont obtenu une libération plus prolongée et des quantités plus importantes de risedronate au niveau osseux grâce à leurs particules qui ont été administrées par voie pulmonaire. Baek *et al.* (2011) ont conclus que des microparticules contenant du chitosane ont donné une augmentation de trois fois du captage de l'alendronate par les cellules Caco-2 comparées à la forme solution. Les particules qui ont été préparées par Glowka *et al.* (2010) ont assuré une libération prolongée de la calcitonine. En plus, une augmentation de la biodisponibilité a été obtenue. Cependant, d'autres études chez l'Homme pourraient être réalisées pour évaluer davantage l'efficacité de tels vecteurs en clinique. Des travaux de recherche plus récents se sont focalisés sur le développement des particules qui ciblent d'une manière spécifique le tissu osseux. Ces travaux reposent sur l'utilisation de substances chimiques qui présentent une affinité envers l'os comme les tétracyclines, les bisphosphonates et l'œstradiol. Ce ciblage permet d'optimiser davantage l'efficacité des molécules actives et de minimiser leurs effets indésirables.

Références :

Baek, J.S., Kwon, H.H., Hwang, J.S., Sung, H.C., Lee, J.M., Shin, S.C., Kim, Y.R., Cho, C.W., 2011. Alendronate-loaded microparticles for improvement of intestinal cellular absorption. J. Drug Target. 19, 37-48.

Glowka, E., Sapin-Minet, A., Leroy, P., Lulek, J., Maincent, P., 2010. Preparation and *in vitro-in vivo* evaluation of salmon calcitonin-loaded polymeric nanoparticles. J. Microencapsul. 27, 25-36.

Mondal, T., Sunny, M.C., Khastgir, D., Varma, H.K., Ramesh, P., 2012. Poly(llactide-co-ε-caprolactone) microspheres laden with bioactive glass-ceramic and alendronate sodium as bone regenerative scaffolds. Mater. Sci. Eng. C 32, 697-706.

Nasr, M.A., Awad, G.A.S., Mansour, S., Taha, I., Al Shamy, A., Mortada, N.D., 2011. Different modalities of NaCl osmogen in biodegradable microspheres for bone deposition of risedronate sodium by alveolar targeting. Eur. J. Pharm. Biopharm.79, 601-611.

International Journal of Pharmaceutics 445 (2013) 181–195

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Review

Drug carriers in osteoporosis: Preparation, drug encapsulation and applications

K. Miladi[a,b], S. Sfar[b], H. Fessi[a], A. Elaissari[a,*]

[a] University of Lyon, F-69622, Lyon, France; Lyon-1 University, Villeurbanne, CNRS, UMR 5007,LAGEP, CPE, 43 bd. du 11 Nov.1918, F-69622, Villeurbanne, France
[b] Laboratoire de pharmacie galénique, Faculty of Pharmacy of Monastir, Rue Avicenne, 5000 Monastir, Tunisia

ARTICLE INFO

Article history:
Received 14 September 2012
Received in revised form
10 December 2012
Accepted 16 January 2013
Available online 31 January 2013

Keywords:
Osteoporosis
Carriers
Encapsulation
Capsules
Drug release
In vivo
In vitro
Low bioavailability

ABSTRACT

Carriers are largely used to enhance therapy efficiency *via* the encapsulation of active molecules. The encapsulation enhances the stability of drug molecules, improves the targeting properties and prolongs pharmacological activity *via* continuous local release of active molecules. The aim of this review is to report the carrier systems used in osteoporosis therapy. This state of the art research has mainly focused on describing all types of carriers used in this area, their elaboration and properties, the drug characteristics used in such specific application, and drug release and efficiency. In this field, various processes have been used in order to obtain well-defined capsules, spheres and more complex carriers. In this exhaustive review, each process is described, illustrated and discussed.

Contents

1. Introduction

Osteoporosis is by far the most frequent metabolic disease affecting the bone. This debilitating chronic disease is characterized by a low bone mass and a microarchitectural deterioration of bone tissue. This leads to an enhanced bone fragility and risk of fracture, particularly the long bones and the vertebrae. Osteoporosis represents also a serious public health problem as all osteoporotic

* Corresponding author at: Lyon 1 University, CNRS, UMR 5007, Laboratoire d'Automatique et de Génie des Procédés, LAGEP-CPE-308G, 43 bd. du 11 Nov.1918, F-69622, Villeurbanne, France. Tel.: +33 472431841; fax: +33 472431682.
E-mail address: elaissari@lagep.univ-lyon1.fr (A. Elaissari).

0378-5173/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijpharm.2013.01.031

fractures are linked with increased morbidity and also because fractures of the hip and the vertebrae are associated with a significant mortality (Holroyd et al., 2008).

Osteoporosis may affect both sexes but women are more vulnerable for the disease because of an acceleration of bone loss after the menopause. In addition to general nonpharmacological measures (such as ensuring adequate dietary calcium and vitamin D intake, lifestyle modifications like smoking cessation, and exercises and reduction of alcohol intake), conventional treatment options for the disease include the use of antiresorptive therapy or anabolic agents. Antiresorptive therapy include essentially bisphosphonates (BP), hormone replacement therapy (HRT), selective estrogen receptor modulators (SERM) and calcitonin (CT), while anabolic agents comprise parathormone (PTH) and its analogs (Lecart and Reginster, 2011; MacBane, 2011; Roush, 2011; Gennari et al., 2009). The goals of treatment are to prevent fracture, preserve structural bone integrity and to decrease morbidity and mortality related to fractures (Follin and Hansen, 2003).

All the therapies mentioned above present some bioavailability concerns. In order to overcome this shortcoming, many approaches were used to obtain more convenient formulations with either better oral bioavailability or providing better patient compliance such as injectable sustained drug delivery systems. Therefore, attention was paid to deliver drugs in carrier systems.

Processes used for the preparation of these carriers include multiple techniques such as emulsion solvent process, spray drying, emulsion polymerization, emulsion solvent diffusion, rapid expansion of supercritical solutions, and ionic gelation. These various techniques led to the obtaining of a multitude of pharmaceutical forms ranging from polymeric microparticles and nanoparticles to nanocrystals and liposomes. Recently, an oral formulation of CT (Ostora® developed by Tarsa Pharmaceuticals) achieved all of the efficacy endpoints in a phase III clinical trial. Obtained results showed that reductions in bone resorption markers with the novel oral formulation were greater than those observed in nasal spray or placebo. This revolutionary event may open wide horizons for the development of other oral pharmaceutical forms of peptides as, to our knowledge, few peptides reached successfully this advanced clinical phase as oral drug delivery systems (Binkley et al., 2012).

2. Challenges in osteoporosis therapy

Therapeutic options that can be used in osteoporosis include antiresorptive agents and anabolic therapy. Antiresorptive agents include BP, HRT, SERMs and CT. BP are still used first-line for treatment and prevention although they present very low oral bioavailability and many side effects related especially to the gastrointestinal tract, with esophageal irritations being the most frequent manifestation. In fact, oral absorption ranges from about 0.7% (for alendronate and risedronate) to only 6% (for etidronate and tiludronate). Furthermore, drug absorption is reduced by food and by products containing calcium or polyvalent cations (Cremers et al., 2005).

HRT comprises the use of estrogens or progestins. Again, oral bioavailability is poor for natural estrogens and progestins because of the important first pass metabolism exerted by the liver and the gastrointestinal tract (Brar, 2010; Zaghloul et al., 2005; Christiansen, 1996). Raloxifene, a SERM which is given orally as tablets presents a low bioavailability (absolute bioavailability less than 2%) due to its poor solubility in biological fluids and its extensive first pass metabolism. High-fat meal can increase absorption but without actual clinical impact (Thakkar et al., 2011; Pickar et al., 2010; Wempe et al., 2008; Hochner-Celnikier, 1999). CT is given either by subcutaneous injection or intranasally as a spray. Following parenteral administration, CT has a short half-life (15–20 min) which needs frequent administrations. On the other hand, nasal spray provided a bioavailability of only 10–25% compared with the parenteral form.

Anabolic therapy represents another pharmacological approach for osteoporosis management. It includes PTH and its analog teriparatide. The latter is administered by subcutaneous daily injections which may compromise patient compliance because of the chronic nature of osteoporosis. Thus, development of an oral drug delivery system of teriparatide will be interesting but must challenge the acid-induced hydrolysis of the active pharmaceutical ingredient (API) in the stomach and its poor membrane permeability (Goldberg and Gomez-Orellana, 2003).

3. What are the main advantages of using carriers compared to conventional aspect based on use of molecules solution?

The shortcomings of pharmacological therapy and the crucial achievements related to the formulation of carrier systems, especially, the enhancement of the bioavailability of various active molecules (Raffin et al., 2012; Vural et al., 2011; Sahoo et al., 2007), led to attempts to investigate their application in osteoporosis. The use of carriers as drug delivery systems provides advantages over a simple solution of active molecules as they can protect drug from inactivation (by light or enzymatic attack) and also reduce its toxicity (Khachane et al., 2011; Mazzaferro et al., 2012; Tammam et al., 2012; Barratt, 2003). Moreover, encapsulation allows the masking of unpleasant taste of some drugs. Enhancement of the therapeutic efficacy may also be obtained as biodistribution of the active molecule depends no longer on its own physicochemical properties but on carrier's ones (Gagliardi et al., 2012; Heneweer et al., 2012; Herrero et al., 2012; Mora-Huertas et al., 2010). In fact, carriers may, compared to drug solutions, provide better membrane absorption and targeting of the drug to the tissue where the pharmacotherapeutic action takes place. Reproducible and long-term release of the drug at the target site is then provided (Cintra e Silva et al., 2012; Levchenko et al., 2012; Poletto et al., 2012; Wang et al., 2012a; Cenni et al., 2008; Sahoo et al., 2007).

4. Methods used for the preparation of carriers

Various methods were used for the preparation of carriers depending on their nature: particulate carriers (with vesicular or spherical form) or lipid carriers like liposomes which are vesicles composed of concentric lipid bilayers (Beija et al., 2012). Used techniques for the formulation of particulate carrier systems are classified in two categories: techniques based on the use of preformed polymers and methods relying on polymerization of monomers. Techniques based on the use of preformed polymers comprise solvent evaporation, nanoprecipitation, salting out, dialysis, spray drying, emulsion solvent diffusion and emulsion coacervation.

On the other hand, methods relying on the use of the polymerization of monomers include emulsion polymerization and interfacial polymerization. Liposomes are obtained by other methods such as lipid film hydration, reverse phase evaporation, solvent injection, freeze–thaw and the microfluidization technique (Meure et al., 2008). The adsorption of drug molecules to preformed carriers' surface is also an alternative. In this study, we will focus on methods used for preparation of carriers intended to the treatment of osteoporosis. For further data about all the above-cited preparation methods, interested reader may refer to reviews of Rao and Geckeler (2011) and Mora-Huertas et al. (2010).

Eight of the methods cited above were used to prepare carriers designed for osteoporosis treatment: emulsion solvent

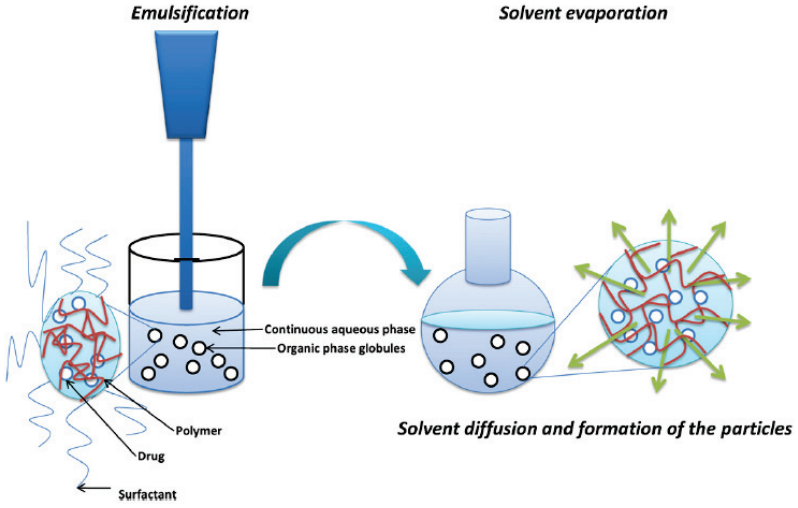


Fig. 1. Emulsion solvent evaporation method.

evaporation, spray drying, ionic gelation, lipid film hydration, emulsion solvent diffusion, emulsion polymerization, rapid expansion of supercritical solutions and adsorption of API on particles surface.

4.1. Emulsion solvent evaporation method

Emulsion solvent evaporation technique is widely used in the field of particulate carriers' development. This method was first developed by Vanderhoff et al. (1979). It consists of the formation of a simple or a double emulsion and the subsequent evaporation of the organic solvent which leads to the precipitation of the polymer and the obtaining of the particles (Fig. 1).

More precisely, the polymer is first dissolved in a volatile and nonmiscible organic solvent such as chloroform, ethylacetate or dichloromethane. This organic phase is then dispersed by high speed homogenization or by sonication in an aqueous phase that contains a surfactant. Once an oil-in-water (o/w) emulsion is obtained, the evaporation of the organic solvent allows its diffusion to the outer phase leading to the formation of the particles. This method of simple emulsion solvent evaporation is generally used for the encapsulation of hydrophobic drugs (O'Donnell and McGinity, 1997). Conversely, if the active pharmaceutical ingredient is hydrophilic, the double emulsion technique will be more suitable: in this case, another step consisting of the dispersion of the primary emulsion (generally a w/o emulsion) in a second aqueous phase is necessary before organic solvent evaporation (Giri et al., 2012).

In both cases, evaporation of the organic solvent is obtained by stirring the emulsion on room temperature or under high temperature and low pressure conditions. The obtained particles can be then harvested by ultracentrifugation or filtration then washed and lyophilized. Interested reader may refer to the study of Rosca et al. (2004) for further details about the mechanism of formation of particulate carriers by emulsion solvent evaporation technique. Table 1 shows antiosteoporotic formulations obtained by the emulsion solvent evaporation process. These particles consisted essentially of poly(lactide-co-glycolide) (PLGA) based microspheres and BP were the most common active ingredients to be encapsulated (9 formulations out of 17).

Mondal et al. (2012) prepared poly(lactide-co-caprolactone) based microspheres loaded with alendronate sodium (ALD) and bioactive glass ceramic. The encapsulation efficiency (EE) of the drug was found to be 11.5% and 14% for 50 mg and 100 mg drug loading, respectively. Although, the double emulsion method is an alternative for hydrophilic drug entrapment (such as ALD), tendency of the drug to escape to the outer phase may compromise EE. In fact, Mondal et al. (2012), by using the simple emulsion method, found higher EE than those reported by Nafea et al. (2007) (0.2%) who opted for the double emulsion method.

In addition, Mondal et al. (2012) evaluated the microspheres he prepared by studying the *in vitro* toxicity in L929 cells and performing the MTT assay (3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium bromide test). Obtained data indicated the noncytotoxic nature of the formulation. Additional studies proved that loaded microspheres promoted cell adhesion.

Nasr et al. (2011) encapsulated risedronate sodium (RS), another hydrophilic BP, in poly(lactide-co-glycolide) (PLGA) microspheres by the double emulsion technique. To overcome the diffusion of the drug from the inner phase to the outer aqueous phase, an osmogen agent, NaCl, was added to the outer phase. In fact, a general increase in the EE was noticed when the amount of NaCl in the outer phase was increased. Authors concluded that the presence of NaCl in the external phase provided an effective mechanical barrier to drug transfer and kept the internal aqueous phase droplets small. In addition, it was thought that NaCl would speed up the process of polymer precipitation and would hamper diffusion of the drug by a saturation effect due to its high solubility in water (Perugini et al., 2001; Uchida et al., 1996).

Microspheres prepared by Nasr et al. (2011) were regarded to be safe after they had been subjected to MTT assay. In addition, *in vivo* tests were conducted on rats after lung delivery of the formulation. Examination of rat lung tissue revealed no histological alteration after 24 h of administration of the microspheres. In addition, results obtained after the radiolabeling of RS prior to its administration either intravenously or by the orotracheal route were encouraging. In fact, compared to the injectable route, microparticulate form ensured a sustained delivery and provided higher amounts of deposited RS in bone.

Table 1
Formulations prepared by the emulsion solvent evaporation method.

Pharmaceutical formulation	Polymer or material	API	Particles size	Route	Efficacy studies	References
Microspheres	Poly(lactide-co-caprolactone)	Alendronate sodium Bioactive glass ceramic	–	Local delivery	*In vitro*	Mondal et al. (2012)
Microspheres	PLGA	Risedronate sodium	3.6–10 μm	Lung delivery	*In vitro* *In vivo*	Nasr et al. (2011)
Microspheres	Eudragit® L100-55 and PEO, Eudragit® L100-55 and carbomer, Eudragit® L100-55 and Chitosan	Alendronate sodium	750–2200 μm	Oral	*In vitro*	Baek et al. (2011)
Microspheres	Calcium phosphate	Alendronate sodium	163–195 μm	Local delivery	*In vitro* *In vivo*	Kim et al. (2010)
Microspheres	PLA, PLGA, PLA-PEG	3-Ethyl-4-(4-methylisoxazol-5-yl)-5-(methylthio) thiophene-2-carboxamide	10–50 μm	Intramuscular injection	*In vitro* *In vivo*	Umeki et al. (2010)
Nanoparticles	PLGA Eudragit RS PO	Calcitonin	441– 609 nm	Injectable	*In vitro* *In vivo*	Glowka et al. (2010)
Microspheres	Poly(β-hydroxybutyrate-co-β-hydroxyvalerate)	Alendronate and hydroxyapatite	214 μm	–	*In vitro*	Huang et al. (2009)
Microspheres	PLGA	Alendronate and hydroxyapatite	77.6–132.56 μm	Implantation	*In vitro*	Shi et al. (2009a)
Microspheres	PLGA	Alendronate sodium	36.81– 74.36 μm	Implantation	*In vitro*	Samdancioglu et al. (2006)
Microparticles	PLGA, PLA	Estradiol	72 μm	Implantation	*In vitro*	Zaghloul et al. (2005)
Microspheres	PLGA	Parathormone	20–50 μm 2–5 μm <1 μm	Implantation	*In vitro* *In vivo*	Wei et al. (2004)
Microspheres	Eudragit P-4135F	Calcitonin	166.2 μm	Oral	*In vitro* *In vivo*	Lamprecht et al. (2004)
Microspheres	PLG-GLU	Pamidronate disodium	87–117 μm	Implantation	*In vitro*	Weidenauer et al. (2003)
Microspheres	PLGA	Ipriflavone	43–53 μm	Local injection	*In vitro* *In vivo*	Perugini et al. (2003)
Microspheres	PLGA	Estradiol	76 μm	Injectable	*In vitro* *In vivo*	Otsuka et al. (2002)
Microspheres	PLGA, PLA	Clodronate	15–113 μm	Local injection	*In vitro*	Perugini et al. (2001)
Microspheres	PGLA, PLA	Progesterone	37–250 μm	–	*In vitro*	Yang and Owusu-Ababio (2000)

PEO, polyethylene oxide; PLG-GLU, poly(lactide-co-glycolide-glucose).

Baek et al. (2011) used the conventional double emulsion method to encapsulate ALD. EE of each formula ranged from 57.33 to 100% depending on the blend of polymers used and on the weight ratio of polymers to ALD. The higher EE were obtained for microparticles based on Eudragit® L100-55 and Polyethylene oxide (PEO) blend for all polymer to ALD ratios and for microparticles based on Eudragit® L-100-55 and Carbomer when polymer to ALD ratios was less than 0.1. Moreover, *in vitro* studies on Caco-2 cells were conducted to estimate intestinal cellular uptake and potential effects of the Eudragit® L100-55 and chitosan based microparticles on Caco-2 cells morphology. According to the obtained results, there was no significant difference between uptake of ALD from ALD solution and PEO-containing microparticles. Chitosan-containing microparticles showed, however, a threefold increase in cellular uptake of ALD compared with an ALD solution. It was also concluded that, although they did not provide higher EE compared to other blends of polymers, Eudragit® L100-55 and chitosan microparticles provided the highest mucoadhesion properties.

Kim et al. (2010) formulated ALD-loaded calcium phosphate (CaP) microspheres by the simple emulsion method. CaP was chosen as calcium cations have strong ionic affinity to ALD anions (Boanini et al., 2007). CaP based carrier systems were obtained before but presented irregular shapes and sizes. In addition, the generally used loading process, which occurs only on the CaP spheres surface, led to low drug contents (Faucheux et al., 2009; Peter et al., 2005; Josse et al., 2004). To circumvent these drawbacks, Kim et al. (2010) used a new *in situ* process which allows the encapsulation of the drug inside the microspheres and not only on their surface. The *in situ* method consists of the preparation and the encapsulation of the drug in only one step rather than two steps as it's used in the initial technique. The obtained data showed EE up to 72% and as low as 11% for the *in situ* technique and the adsorption technique, respectively.

Experiments of release kinetics of ALD from the microparticles were carried in phosphate buffer solution (PBS) at pH7.4 as a release medium and resulting data revealed an initial burst release of ALD followed by a sustained release of the drug over 40 days. Furthermore, higher dissolution kinetics were obtained when the drug loading of the particles increased. Inhibitory effect of the microparticles on osteoclastogenesis was also noticed as they inhibited the differentiation of RAW264.7 cells into osteoclasts even when stimulated with the requisite cytokines, RANKL and M-CSF.

Umeki et al. (2010) prepared different microspheres using various poly(lactic acid) (PLA), PLGA or poly(D,L-lactic acid)-block-poly(ethyleneglycol) (PLA-PEG) polymers then studied the *in vitro* and *in vivo* release of the new potent osteogenic compound 3-ethyl-4-(4-methylisoxazol-5-yl)-5-(methylthio)-thiophene-2-carboxamide from the microparticles. High EE were obtained (approximately from 70% to 80%). PEG ratio in the PLA-PEG did not significantly influence the EE values but a PEG ratio exceeding 25% resulted in the disappearance of the spherical form, which may be explained by the interaction between the strongly hydrophilic

PEG layer and water. Such an interaction expands, in fact, the contact surface area between PEG and water leading to an irregular surface of the microspheres. Efficacy studies indicated that more than 50% of the active remained in each type of microsphere at 12 weeks after incubation. In addition, some PLA and PLA-PEG based microspheres were able to deliver the active over 6 weeks after intramuscular injection in rats.

Glowka et al. (2010) aimed the development of an injectable sustained release formulation of CT and opted for assessing Eudragit® RS, PLGA/Eudragit® RS and PLGA based nanoparticles. The highest EE was obtained with PLGA 50:50 based nanoparticles. This was explained by favorable interaction of positively charged CT with the free carboxyls in the polymer. Data obtained from experiments conducted *in vitro* and *in vivo* confirmed a more extended release for the PLGA based nanoparticles. Indeed, high serum CT levels were obtained after 3 days of subcutaneous administration in rats and achieved bioavailability increased compared to a CT solution.

Solid in oil in water method (S/O/W) was used by Huang et al. (2009) to prepare ALD-loaded poly(β-hydroxybutyrate-co-β-hydroxyvalerate) microparticles incorporated with hydroxyapatite (HA). HA was used for its chemical similarity to the major inorganic component of bone, its excellent biocompatibility and osteoconductivity which enhances bone targeting and allows more sustained release (Shi et al., 2009b; Wang, 2007). *In vitro* release studies in phosphate buffer saline (PBS) showed sustained release over 26 days. In addition, MTT assay executed on rabbit mesenchymal stem proved safety of the carriers.

Shi et al. (2009a) compared ALD-containing PLGA/HA microspheres formulated by simple and double emulsion methods. Obtained results showed EE was significantly improved by the adoption of the simple emulsion technique (more than 85%) compared to the double emulsion technique (less than 40%). Moreover, the formulation of PLGA microspheres loaded with ALD and without the addition of HA resulted in a significant decrease of the EE.

This was explained by the fact that, in single emulsion microspheres, ALD was pre-attached to HA particles and then subjected to the emulsion process. The affinity between HA and ALD was strong enough to prevent ALD loss. Conversely, in the double emulsion method, HA exposure for ALD was blocked by the PLGA-containing organic phase. Prepared PLGA/HA microspheres ensured *in vitro* sustained release of ALD over 30 days. Also, further assays proved that microspheres inhibited macrophage proliferation and enhanced osteoblastic proliferation (Shi et al., 2009a).

Samdancioglu et al. (2006) obtained low EE of ALD when they prepared PLGA-based microcapsules. Even when pH conditions of the outer continuous phase were changed, the highest EE obtained was as low as 7.7%. *In vitro* release studies showed that 58% of ALD was released from the microspheres by the end of the fifth day, while a total dissolution was obtained in only 1 h with an ALD powder. This may be a promising result for eventual local implantation of the particles.

Zaghloul et al. (2005) used various combinations of PLA, PLGA 85:15 and PLGA 75:25 to prepare beta-estradiol microparticles. Interestingly, *in vitro* release studies showed the same profile for all the formulations but PLGA 85:15 provided a more sustainable release with less important initial burst effect which could be suitable for long therapy. Wei et al. (2004) proposed PLGA based microparticles as new drug delivery system for PTH. Prepared microspheres continued to deliver high plasma levels of biologically active PTH after 24 h, however, intact PTH(1–84) and PTH(1–34) have plasma half lives of less than 3 and 11 min, respectively (Codrons et al., 2003). In addition, further *in vivo* studies in rats indicated effective delivery of biologically active PTH from the microspheres.

CT containing microparticles designed for colonic delivery were proposed by Lamprecht et al. (2004) to avoid the required regular injections. The microspheres were obtained by the double emulsion method. Using Eudragit® as polymer provided EE as high as 76.5% and, interestingly, coencapsulation of chitosan resulted in a slight increase of the EE to 80.9%. According to the results of *in vitro* studies, Eudragit® P-4135F kept the leakage of CT at pH 6.8 below 20% within 4 h while a faster release was obtained at pH7.4. On the other hand, *in vivo* studies showed higher pharmacological effect and a fourfold increase of the area above the curve of calcium blood level compared to levels reached by CT solution.

Weidenauer et al. (2003) used the double emulsion technique to encapsulate another BP, pamidronate, to prepare poly(lactide-co-glycolide-glucose) based microparticles. Theoretical drug content was varied from 2 to 10% and resulted in a yield ranging from 81% to 87%. Up to 90% of EE was obtained when drug content was higher than 6%. Authors tried also to avoid the initial burst release obtained for these formulations and obtain parenteral sustained release system. This was achieved by a solid in oil in oil technique (S/O/O). The external oil phase consisted of Span 80 in paraffin oil. This method permitted a decrease in initial release along with an increase in drug loading.

Perugini et al. (2003) encapsulated the lipophilic drug, ipriflavone, in PLGA based microparticles using the simple emulsion technique. The drug to polymer ratio and the polymer molecular weight influenced significantly the EE. It was found that formulation corresponding to a ratio drug to polymer of 1/5 and a polymer molecular weight of 12,000 gave the highest EE (95.8%). *In vitro* release was completed in approximately 1 month while morphometric analysis in rats indicated a slight increase in spongy and total bone mass result at the level of the third molar. This increase was, nevertheless, not statistically significant (Perugini et al., 2003).

Otsuka et al. (2002) estimated the *in vitro* and *in vivo* estradiol release from PLGA microspheres. *In vitro* estradiol release was maintained at a constant rate for 1 month while bone mineral density for rats after the injection of estradiol loaded microspheres was higher than that obtained for the control.

Perugini et al. (2001) adopted double emulsion method to encapsulate hydrophilic clodronate (CLD) in PLGA and poly(D,L-lactide). The effect of several parameters on EE was studied: molecular weight and molar composition of the used polymer, presence of a viscosity increasing agent carboxymethylcellulose (CMC) in CLD solution or of an emulsifier agent (Span 20) in the polymeric solution and the amount of the drug. Some other parameters were kept without change such as the volume ratio between the aqueous and organic phases of the inner emulsion, presence of NaCl in the outer aqueous phase and temperature rising rate during the organic solvent evaporation phase.

Data showed that polymer molecular weight did not significantly influence EE, however, when different PLGA 50:50 having the same molecular weight were used, the more hydrophilic polymer gave higher EE. This may be explained by the hydrophilic nature of the drug.

Although, increasing the viscosity of the inner aqueous phase was used to enhance EE (Ogawa et al., 1988), addition of CMC had not a significant effect on EE of CLD. Similarly, addition of Span 20 in the organic phase did not increase drug EE. CLD microspheres constructed by PLGA and poly(D,L-lactide) (PDLLA) provided modulated *in vitro* release of the drug ranging from 48 h up to 3 months by suitable selection of polymer, composition, additives, and manufacturing conditions (Perugini et al., 2001).

Yang and Owusu-Ababio (2000) studied the influence of some parameters on EE of progesterone in PLGA50:50, PLGA15:85 and PLA based microspheres prepared by the simple emulsion method. Increasing the volume fraction of the emulsion resulted in an increase of the EE from 75% to 84% for volume fractions 9% and

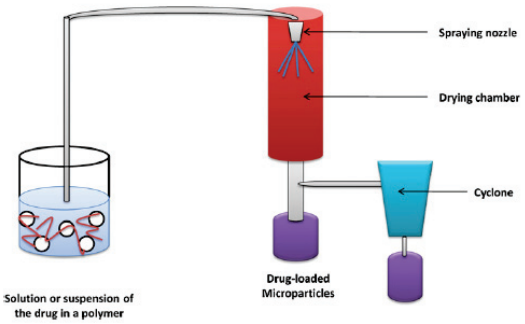


Fig. 2. Spray drying process.

22%, respectively. This was attributed to a decrease in the amount of drug lost to the external aqueous solution when its volume was decreased. EE decreased with increasing the surfactant polyvinyl alcohol (PVA) concentration from 1% to 3% (w/v), but did not decrease with further increase in PVA concentration to 5% (w/v). This decrease in EE was explained by higher loss of the drug to the aqueous phase since the solubility of the drug was found to increase slightly with increasing PVA concentrations. Further studies showed that polymer nature as well as stir speed during the evaporation phase did not influence EE. Additional investigation of the influence of some operating conditions on progesterone release from PLGA 50:50, PLGA 15:85 and PLA based microspheres showed that an increase of the organic phase volume ratio from 9% to 22% resulted in a decrease of the rate of progesterone release (from 65% of progesterone released in 24 h to 50% in 24 h). The latter phenomenon was also observed when stirring speed diminished, whereas increasing PVA concentration led to an increase of the release rate. Furthermore, polymer nature exerted an influence on the release kinetics as the lowest rate of drug release was observed with PLGA 50:50 (Yang and Owusu-Ababio, 2000).

4.2. Spray drying process

Spray drying technique allows the transformation of materials form a liquid to a solid state. This technique was widely used for drying liquid formulations such as solutions, emulsions or suspensions but it gained tremendous interest since its application had been extended to the development of microparticles (Palmieri et al., 2001). As Fig. 2 shows, practically, a solution or a suspension of the drug in a solvent, which contains also the polymer, is sprayed from a nozzle in a hot drying medium. Atomization transforms the liquid stream into fine droplets. Subsequent high surface to volume ratio favors efficient and rapid drying of the droplets and thus, their transformation to particles (Paudel et al., 2012; Call and Sollohub, 2010; Yashwant and Deepack, 2009). When the polymer is not used, the obtained particles are called nanocrystals (Keck and Müller, 2006) or microcrsytals depending on their size but amorphous particles may also be obtained as a consequence of a rapid spray drying process (Paudel et al., 2012). This method has some advantages like its ease of scale up and its mild processing conditions, but it presents some drawbacks such as the high cost of spray dryers. The key parameters that have to be controlled within the process are inlet and outlet temperatures, spray flow and the volume of the air inlet (Yashwant and Deepack, 2009).

Ribeiro Rattes and Oliveira (2007) studied the effect of the inlet temperature on the moisture content of diclofenac sodium microparticles and found that increasing drying temperatures resulted in low moisture content. In addition, the average hydrodynamic particle size was found to increase with an increase of the spray flow. Some formulations prepared by the spray drying technique are shown in Table 2. ALD was used as active in all the formulations to prepare mainly microparticles. Sultana et al. (2012) used spray drying to prepare ALD nanocrystals by adding an antisolvent (organic solvent). Authors used different organic solvents (acetonitrile, ispropyl alcohol, ethanol and acetone) and noticed that the smallest particles were obtained with acetonitrile. Effect of the stabilizer concentration was also studied and data showed that minimum 1% concentration of Poloxomer F68 was required to produce stable nanoparticles. When stabilizer concentration was rised to 20%, large aggregates were obtained.

An experimental design was used to optimize the formulation of nanocrystals. Studied parameters were drug concentration, organic solvent volume, stabilizer concentration and stirring rate. Data showed that an increase of the drug amount resulted in an increase of the particle size. This was explained by an increase of the viscosity of the organic solution subsequent to the increase of the drug concentration, which hindered the diffusion process between solvent (water) and antisolvent. Crystal growth, therefore, took place and led to an increase of the particle size. Conversely, the antisolvent volume and particle size were inversely correlated. This was explained by an increment in antisolvent volume that increased the diffusion of the precipitated nuclei from solvent to antisolvent, which led to a decrease of the particles size.

The increase of the stirring rate from 300 to 900 rpm resulted in decrease in the average particle size. This may be explained by an increase of the rate of diffusion of nuclei into antisolvent. These nanocrystals were prepared in order to increase bioavailability of ALD subsequent to lung delivery. Respirable fraction of optimized nanocrystals (43.85%) was significantly higher when compared with commercial ALD (17.60%) (Sultana et al., 2012).

Cruz et al. (2011) prepared microparticles loaded with ALD intended to lung delivery. Spray drying process was based on aqueous ethanol feedstock. The addition of a nonaqueous solvent in feedstock is advantageous because it generally increases both the outlet temperature and the yield and decreases moisture content and particle size (Seville et al., 2007). A dispersibility enhancer (leucine) and a pore-forming agent (ammonium bicarbonate) were added to the formulation. Leucine concentration influenced

Table 2
Particles prepared by the spray drying process.

Pharmaceutical formulation	Polymer or material	API	Particles size	Route	Efficacy studies	References
Nanoparticles	Stabilizers	Alendronate sodium	44.11 nm	Lung delivery	*In vitro*	Sultana et al. (2012)
Microparticles	Ammonium bicarbonate and leucine	Alendronate sodium	0.93–2.29 μm	Lung delivery	*In vitro*	Cruz et al. (2011)
Microparticles	Eudragit S100 and Methocel K15M	Alendronate sodium	13.8 μm	Oral	*In vitro* *In vivo*	Cruz et al. (2010b)
Microparticles	Eudragit S100 and a blend with Methocel E4M	Alendronate sodium	7.3–17.1 μm	Oral	*In vitro*	Cruz et al. (2010a)
Microspheres	Eudragit S100 and Methocel F4M Eudragit S100 and Methocel K100LV	Alendronate sodium	11.7 μm 8.4 μm	Oral	*In vitro*	Cruz et al. (2009)

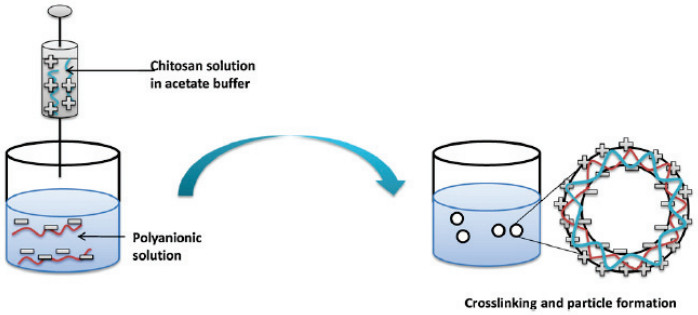


Fig. 3. Ionic gelation method.

particles diameter. In fact, an increase of leucine concentration led to an increase of particle diameter. Formulation prepared with 10% leucine, displayed the best aerosolization properties with a fine particle fraction around 80% and an alveolar fraction around 42%. *In vivo* lung toxicity evaluation by bronchoalveolar lavage studies showed no significant increase of indicators for acute lung injury by ALD microparticles after intratracheal administration. After administration to dogs, bioavailability of 6.23% was obtained compared to 1.6% bioavailability achieved by the oral delivery (Lin et al., 1991). This substantial enhancement arises from the good alveolar deposition of the powder (Cruz et al., 2011).

Gastroresistant microparticles containing ALD were also formulated by Cruz et al. (2010b). The microparticles were based on a blend of Eudragit® S100 and Methocel® K15M. Gastroresistance of the particulate form was proved by *in vitro* dissolution studies. The antiresorptive activity of the particles was confirmed by *in vivo* studies in rats. In addition, rats which received solution of ALD presented an ulcer lesion index (ULI) of 6.5 and a total area of lesion of about 4 mm^2 whereas rats that received microparticles had much lower ULI of 1.33 and a total area of lesion of 1.12 mm^2. Consequently, microencapsulation of ALD reduced its gastrointestinal side effects which may be promising to enhance patient adherence and thus, therapeutic efficacy (Cruz et al., 2010b).

Cruz et al. prepared other microparticles based on Eudragit S100 and blends of Eudragit® S100 with Methocel® E4M (Cruz et al., 2010a), Methocel® F4M or Methocel® K100LV (Cruz et al., 2009). High EE values ranging from 80.7% to 100% were obtained. *In vitro* studies confirmed the gastroresistance of the microparticles. *In vivo* studies conducted for some of these formulations confirmed the protection conferred the rat stomachs against ulcer formation by ALD (Cruz et al., 2010a).

4.3. Ionic gelation method

Ionic gelation is considered as a mild process as use of toxic organic solvents and surfactants is avoided. The technique is based on electrostatic interaction between oppositely charged polymer and a polyelectrolyte. Practically, a solution of the charged polymer is added dropwise under stirring to an oppositely charged polyelectrolyte which causes the cross-linking of the two entities and thus, the obtaining of the particulate form (Fig. 3) (Doustgani et al., 2012; Fan et al., 2012). As shown in Table 3, ionic gelation was widely used for the preparation of chitosan particles. The electrostatic interaction may occur between positively charged chitosan (in an acidic medium) and negatively charged arabic gum (Avadi et al., 2010) or tripolyphosphate (TPP) (Jafarinejad et al., 2012; Li et al., 2011).

Interesting work has been reported by Fan et al. (2012) by preparing nanoparticles using the ionic gelation method. It was demonstrated that changing mass ratio of chitosan over TPP gave various particles sizes ranging from 133 to 237 nm. In the same work, it was concluded that varying the concentration of acetic acid (used to solubilize chitosan and to provide acidic medium) from 0.1 to 0.5 mg/ml did not significantly change the particle size but affected dramatically polydispersity index of the particles which ranged from 0.026 to 0.204 with subsequent consequences on the uniformity of the particles size. The effect of the pH of the chitosan solution was also studied by Shu and Zhu (2002) who noticed that protonation degree of chitosan decreased rapidly from 100% to 0% when the pH changed from 4.7 to 8, which no longer allows the triggering of the ionic gelation process when polyanion TPP is added.

Many other parameters may influence the properties of the obtained particles such as, stirring rate and the ambient temperature at which the process of cross-linking takes place (Jafarinejad et al., 2012). Table 3 shows different particulate formulations designed by the ionic gelation process. Used polymers include chitosan and chitosan derivatives and the prepared drug delivery systems ranged from nanoparticles of less than 330 nm to 0.9 mm-sized beads (Makhlof et al., 2010; Zhao et al., 2010; Aydin and Akbuga, 1996).

Makhlof et al. (2010) opted for ionic gelation method to formulate nanoparticles containing CT. Authors compared glycolchitosan

Table 3
Particulate carriers formulated by the ionic gelation technique.

Pharmaceutical formulation	Polymer or material	API	Particles size	Route	Efficacy studies	References
Nanoparticles	Glycolchitosan Glycolchitosan-thio-glycolic acid	Calcitonin	245 nm 332 nm	Lung delivery	*In vitro* *In vivo*	Makhlof et al. (2010)
Nanoparticles	N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride	Parathormone related protein1-34	100–180 nm	–	*In vitro*	Zhao et al. (2010)
Beads	Chitosan	Calcitonin	0.9 mm	–	*In vitro*	Aydin and Akbuga (1996)

(GCS) to a novel thiomer derivative of glycolchitosan formed by conjugation with thioglycolic acid (glycolchitosan–thioglycolic acid: GCS–TGA). The latter was designed to improve mucoadhesive properties of the particles (Bernkop-Schnürch et al., 2006). Polymer nature affected drug EE as GCS–TGA nanoparticles provided higher CT entrapment (63.6%) than GCS nanoparticles (54.2%). This was explained by more disulfide bonding between CT and GCS–TGA (Sakloetsakun et al., 2009).

The pulmonary mucoadhesion and toxic effects of the nanoparticles were assessed after intra-tracheal administration to rats. Obtained data showed about two-fold increase in the association of the GCS–TGA nanoparticles to the lung tissue compared to GCS nanoparticles. This effect may be explained by the attachment of thiol groups (provided by TGA) to the lung tissue. Toxicity studies revealed the biocompatibility of both formulations with lung tissue. In addition, absorption of CT was evaluated *in vivo* by direct delivery of a CT solution or nanoparticles to the lungs of anesthetized rats. GCS and GCS–TGA nanoparticles prolonged significantly the hypocalcemic effect of CT with phamacological bioavailability of 27% and 40%, respectively. Such an enhanced absorption could be explained by partial protection of the drug from enzymatic degradation and again by the permeation and mucoadhesive effects exerted by the polymers (Makhlof et al., 2010).

Zhao et al. (2010) studied the *in vitro* release of PTH related protein PTHrP1-34 (a malignacy induced peptide with antiosteoporotic properties) from N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (HTCC) nanoparticles. Polymer to TPP ratio is a crucial parameter to obtain nanoparticles. When HTCC to TPP ratio was about 5:1 to 2:1, nanoparticles were formed. Conversely, very high polymer concentrations or high TPP amounts led to no nanoparticles formation or to the obtaining of aggregates with large size, respectively. Authors studied also the effect of active amount, polymer amount on EE and drug loading. Increasing active concentration decreased EE and increased loading capacity. When polymer concentration had risen from 0.5 to 1.5 mg/ml, EE increased from 58.5% to 82.3%. However, further increase of polymer concentration decreased EE. *In vitro* studies showed that this novel drug delivery system provided sustained release of the antiosteoporotic agent over 4 days (Zhao et al., 2010). Ionic gelation method was also used by Aydin and Akbuga (1996) to formulate chitosan beads containing CT. Viscosity of the chitosan sample was crucial in the formation of beads. EE was higher than 50% and was not influenced by initial drug amount. Investigation of kinetic properties of the formulation *in vitro* showed that release of CT could be sustained for 24 days.

4.4. Lipid film hydration method

Lipid film or thin film hydration method is used for the preparation of liposomes which consists of enclosed vesicles composed by phosphatidylcholine and cholesterol bilayers. Liposomes enable the loading of hydrophilic drugs in their inner aqueous phase as well as the insertion of lipophilic drugs within the lipid bilayers. As Fig. 4 shows, the technique consists of the dissolution of the lipophilic excipients in an organic solvent mainly ethanol, methanol, chloroform or diethylether in a round-bottom flask. The obtained solution is evaporated under reduced pressure until the formation of a multilayered film of phospholipids which will be subsequently hydrated by water or a buffer solution to obtain a crude dispersion of liposomes (Laouini et al., 2012; Chen et al., 2009; Meure et al., 2008). Sonication, homogenization or extrusion may also be performed to ensure size reduction and uniformity of the particles. In the recently published work of Park et al. (2011), it was concluded that lipid ratio and the nature of the aqueous phase affected the size of the obtained liposomes. In fact, particle sizes ranged from 265 nm to 335 nm when three lipid ratios were tested. Furthermore, sizes of liposomes hydrated in phosphate buffer saline (PBS), 5% dextrose, or 10% sucrose were about 202 nm, 258 nm, and 222 nm, respectively.

Table 4 summarizes some formulations prepared by the lipid film hydration method. Lipid film hydration method was used to prepare liposomes encapsulating either ethinylestradiol or CT. Lu et al. (2011) prepared liposomes loaded with ethinylestradiol. Cholesterol is commonly used for liposomes formulation for many reasons. In fact, it improves the fluidity of the bilayer membrane, reduces the permeability of water soluble molecules through the membrane, and enhances the stability of bilayer membrane in the presence biological fluids such as blood or plasma (Vemuri and Rhodes, 1995). In spite of these advantages, authors wanted to avoid the use of cholesterol because it was shown that it can reduce

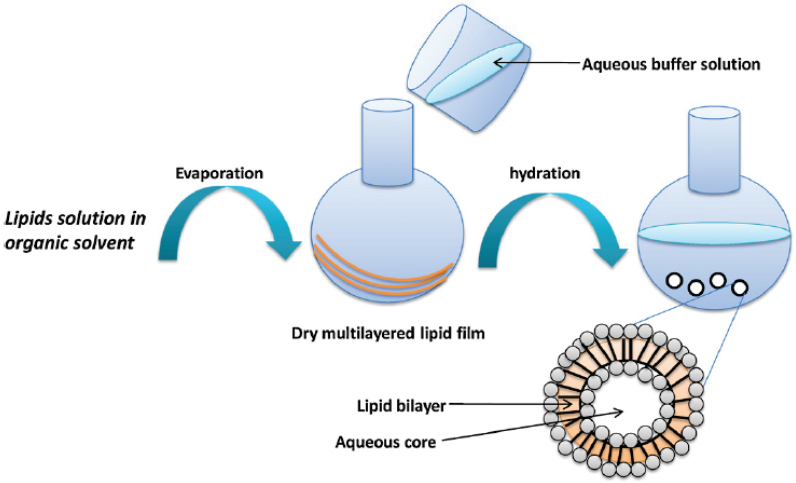


Fig. 4. Lipid film hydration method.

Table 4
Carriers prepared by the film hydration method.

Pharmaceutical formulation	Polymer or material	API	Particles size	Route	Efficacy studies	References
Liposomes	Egg phosphatidylcholine, cholesterol	Ethinylestradiol	175.9–235.1 nm	Injectable	*In vitro* *In vivo*	Lu et al. (2011)
Carbopol and chitosan coated liposomes	DPPC, DCP	Calcitonin	–	Oral	*In vitro* *In vivo*	Takeuchi et al. (2003)
Liposomes	DOPC, cholesterol	Calcitonin	990–1411 nm	Nasal delivery	–	Law and Shih (2001)

DPPC, dipalmitoylphosphatidylcholine; DCP, dicetylphosphate; DOPC, dioleylphosphatidylcholine.

EE of active pharmaceutical ingredients (Mohammed et al., 2004). The lipophilic active, ethinylestradiol, was used as alternative and obtained results confirmed higher EE for noncholesterol containing liposomes. Lu et al. (2011) investigated also the effects of the ethinylestradiol liposomes on ovariectomized rat osteoporosis. At termination of 1 month of intraperitoneal administration of either free (FET) or encapsulated ethinylestradiol (EET), higher increase of bone mineral density was achieved in the EE group (37.8%) than in the FE group (20.3%). These data proved superiority of the EET over the free form in the management of ovariectomized rat osteoporosis.

Takeuchi et al. (2003) coated liposomes with carbopol and chitosan. Such polymers were used as coating materials in order to increase the mucoadhesion of these liposomes designed for oral delivery and thus promoting their absorption through the gastrointestinal mucosa (Grabovac et al., 2005). Authors compared *in vitro* adhesiveness to rats' intestine and *in vivo* activity of different liposomes: carbopol-coated liposomes (CP-LIP), chitosan-coated liposomes (CS-LIP), positively charged uncoated liposomes and negatively charged uncoated liposomes. Obtained results showed that highest adhesion effect was achieved by CS-LIP then CP-LIP then positively charged uncoated liposomes.

Negatively charged uncoated liposomes possessed the lowest adhesion effect. On the other hand, *in vivo* studies showed that overall pharmacological effect of CS-LIP and CP-LIP assessed by the area under the plasma calcium concentration curve was significantly higher than of CT solution and 2.4 and 2.8 times higher than that of negatively and positively charged uncoated liposomes (Takeuchi et al., 2003).

Law and Shih (2001) formulated different charged liposomes (positive, neutral and negative charge). To obtain such liposomes, authors added charge inducing excipients such as, sterylamine (for cationic charge) and phosphatidylserine (for anionic charge). Then, effects of liposomal charge characteristics, molar ratio of charge-inducing agent, and pH of the medium on the EE were studied. EE of CT increased when initial drug amount increased. Charge of liposomes had also a crucial impact: negatively charged liposomes possessed the highest EE followed by neutral then by positively charged liposomes. This was explained by the positive charge of CT which bound to liposomes by electrostatic interaction.

Similarly, the increase of molar ratios of phosphatidylserine from 0.075 to 0.3 resulted in an increase of EE due to the increase of the negative charge of the liposomes. In contrast, the increase of molar ratios of sterylamine in the formulae showed a decrease of EE. Moreover, EE of CT in positively charged liposomes at pH 7.4 was greater than that at pH 4.3. It seems that, at high pH, more amino groups of both sterylamine and CT were deprotonated, causing less electrostatic repulsion between the liposomes and CT molecules and thus, much higher EE. *In vitro* release kinetics of CT showed that positively charged liposomes released more CT, suggesting that electrostatic repulsion played a crucial role in leakage of the active molecule from the liposomes.

4.5. Emulsion solvent diffusion method

This technique was developed by Quintanar-Guerrero and Fessi (1996). Three liquid phases are needed: an organic phase, an aqueous phase and a dilution phase. The organic solvent contains the polymer and eventually, the hydrophobic drug. The aqueous phase comprises the aqueous dispersion of the stabilizing agents, while the dilution phase is usually a large volume of water.

Both, the organic and the aqueous phase must be mutually saturated after use to ensure the subsequent obtaining of thermodynamically equilibrated emulsion. Once the organic phase is emulsified in the aqueous phase under high-speed homogenization, the addition of an excess of water enables the diffusion of the organic solvent from the dispersed phase resulting in precipitation of the polymer and the formation of the particles (Fig. 5). Commonly used polymers in this method include polycaprolactone (PCL), polylactide (PLA) and Eudragit® (Mora-Huertas et al., 2010). A modified solvent diffusion based method was also proposed for the encapsulation of hydrophilic molecules by using an aqueous inner phase (Ma et al., 2001).

Operating conditions affecting the size of the particles obtained were largely reviewed by Mora-Huertas et al. (2011). They include external/internal phase ratio, emulsification stirring rate, volume of water for dilution and the temperature of added water. Emulsion solvent diffusion method was used for antiosteoporotic formulations, mainly to prepare nanoparticles as Table 5 shows. In an interesting study, Mittal et al. (2007) used different polymers to encapsulate estradiol in nanoparticles based on PLGA 50:50, PLGA 65:35 and PLGA85:15 of various molecular weights. EE did not follow a regular pattern. First, it decreased from 51.3% to 34.5%, as polymer molecular weight was increased from about 14,500 to 85,000 Da; thereafter, a significant increase was observed to 67.8% as molecular weight was increased to 213,000 Da. This was explained by two phenomena. Firstly, an increase in viscosity on increasing the molecular weight might have decreased the diffusion rate of solvent into the external aqueous phase. Slow polymer precipitation gave drug molecules more time to diffuse to the aqueous phase, resulting in low EE. Secondly, further increase of the

Table 5
Particles prepared by the emulsion solvent diffusion method.

Pharmaceutical formulation	Polymer or material	API	Particles size	Route	Efficacy studies	Reference
Nanoparticles	PLGA	Estradiol	118.2–129 nm	Oral	*In vitro* *In vivo*	Mittal et al. (2007)
Nanoparticles	PLGA	Estradiol	148.3 nm and 410.9 nm	Oral	*In vitro* *In vivo*	Hariharan et al. (2006)
Nanospheres	PLGA	Elcatonin	250 and 700 nm	–	*In vitro*	Kawashima et al. (1998)

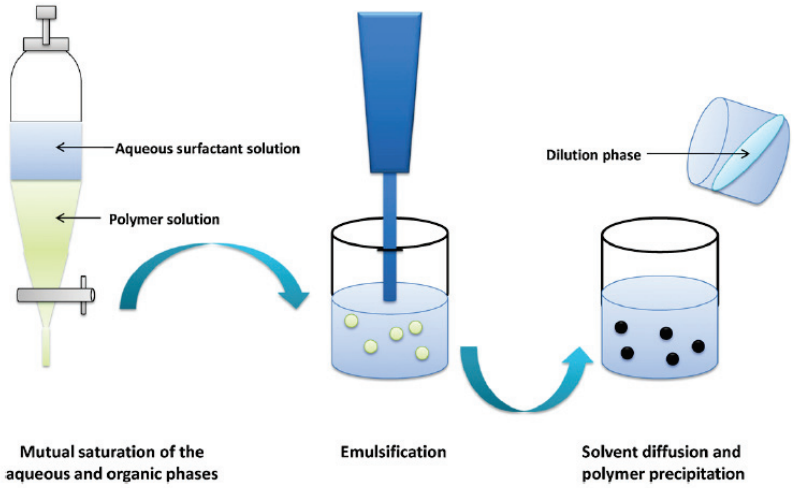


Fig. 5. Emulsion solvent diffusion.

polymer molecular weight resulted in strong hydrophobic interactions of polymer molecular chains and the lipophilic drug.

PLGA 50:50 was found to have the lowest EE. While there was a significant increase in the EE of PLGA 65:35, no difference was observed between PLGA 65:35 and PLGA 85:15. In fact, when the lactide content of PLGA copolymer was increased in PLGA, hydrophobicity of copolymer also increased. This led to increased solid-state solubility of hydrophobic drug in the polymer, resulting in increased EE as compared to that obtained with PLGA 50:50.

Mittal et al. (2007) investigated also the effect of polymer molecular weight and copolymer composition on *in vitro* and *in vivo* release behavior of estradiol from the nanoparticles intended to oral delivery. *In vitro* drug release was shown to decrease with an increase in the molecular weight and lactide content of the polymer. All PLGA nanoparticles produced detectable blood levels for 5–11 days while only one day profile was obtained for pure drug. In addition, the *in vivo* performance of the nanoparticles depended on particles size, polymer molecular weight and copolymer composition. For instance, the highest area under the plasma concentration (AUC) per time curve was obtained with PLGA 50:50 of 45,000 Da molecular weight based nanoparticles (1936.79 ng h/ml) which was significantly higher than the AUC of pure drug oral suspension (190.51 ng h/ml). Particle size also dictated the fate of nanoparticles *in vivo*. In fact, particles made of 213,000 Da PLGA (with particle size of 155 nm), which were thought to show much sustained release due to the very high molecular weight, released the drug only for 6 days. However, 11 days release profile was achieved by PLGA 65:35 nanoparticles (particle size 126 nm) (Mittal et al., 2007).

Hariharan et al. (2006) investigated the possibility of enhancing estradiol oral bioavailability by incorporating the active in PLGA nanoparticles. Particles were formulated by using either PVA or didodecyldimethylammonium bromide (DMAB) as stabilizer, which led to negatively (size 410.9 nm) or positively (size 148.3 nm) charged particles, respectively. Effects of surfactant concentration and initial drug amount on EE were investigated. It was shown that DMAB concentration did not significantly affect EE. It was also observed that, with both stabilizers, there was an increase in EE with an increase in initial drug amount. *In situ* intestinal uptake studies were conducted in rats to confirm the uptake of particles from the intestine. It was shown that higher uptake was obtained with DMAB stabilized nanoparticles than PVA stabilized nanoparticles but the highest uptake was provided by pure form of drug. Furthermore, *in vivo* studies conducted by oral administration to rats of both encapsulated and pure estradiol gave more interesting results. Pure drug blood profiles indicated short circulation times and drastic fall in blood levels, while estradiol was detected in blood for 7 and 2 days from DMAB and PVA stabilized nanoparticles, respectively. These data were explained by smaller size and the positive charge of DMAB stabilized nanoparticles compared with PVA stabilized nanoparticles, which allows them to better interact with mucin and to prolong circulation time. Therefore, authors concluded that key parameters for enhancing estradiol bioavailability consist on developing particles with positive charge, small size and high polymeric encapsulation. Kawashima et al. (1998) encapsulated elcatonin, a CT derivative, in PLGA nanoparticles. It was shown that emulsion diffusion in oil improved EE of the highly hydrophilic active molecule compared to emulsion diffusion in water.

4.6. Emulsion polymerization method

Emulsion polymerization technique has been largely used to produce latexes. There are two kinds of emulsion polymerization processes depending on the nature of the continuous phase of the emulsion: continuous organic phase methodology and continuous aqueous phase technique. As shown in Fig. 6, the particle polymeric shell is obtained from a monomer that undergoes a polymerization process. The latter process begins after the addition of an initiator that may be an ion or a radical but a monomer itself can be transformed to an initiator following the application of a high-energy radiation such as gamma-radiation, ultraviolet and strong visible light (Pinto Reis et al., 2006). Polyacrylamides, poly(methyl-methacrylates), poly(ethyl-cyanoacrylates) and poly(butyl-cyanoacrylates) are among polymers that can be produced by this method (Rao and Geckeler, 2011). Parameters that manage the properties of the obtained particles include monomer concentration, surfactant nature and concentration and the pH of the polymerization medium.

Tolue et al. (2009) noticed a variation of the shape of acrylonitrile–styrene–acrylate structural latexes prepared by emulsion polymerization technique after changing the nature of

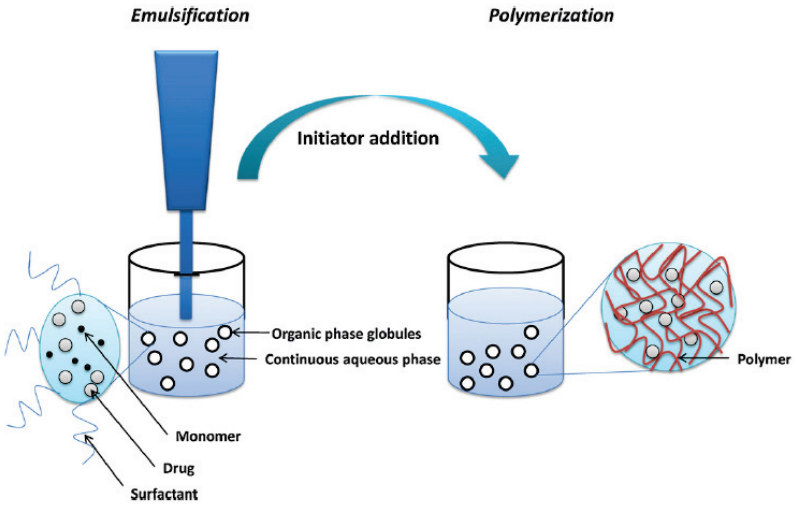


Fig. 6. Emulsion polymerization technique.

the surfactant. In fact, a hemispherical form was obtained when using the ionic surfactant sodium dodecylsulfate, while raspberry and core–shell structures were described for latexes prepared with the nonionic surfactant nonylphenol ethoxylated polyethylene glycol. Temperature of reaction medium seems also to affect particles. Wu et al. (2011) prepared n-butyl-acrylate particles by the emulsion polymerization technique and found that an increase of the reaction temperature from 20 °C to 70 °C resulted in a decrease of the particle size. The method of emulsion polymerization was also used by Tasset et al. (1995) to prepare poly(isobutyl-cyanoacrylate) nanoparticles loaded with CT. The percentage of binding of CT to the prepared nanoparticles was 97.9%. This was explained by high affinity of CT to poly(isobutyl-cyanoacrylate). Aminogroups of CT form, in fact, a covalent binding with poly(alkylcyanoacrylates). These particles released CT *in vitro* over 5 h. Furthermore, intravenous injection of nanoparticles in rats induced a similar profile in the serum calcium concentrations as the intravenous injection of free CT. Conversely, after a subcutaneous administration, nanoparticles acted as a sustained release system for CT: hypocalcemic activity was maintained for 24 h after subcutaneous injection. In addition, more important hypocalcemic than free CT was observed.

4.7. Rapid expansion of supercritical solutions technique (RESS)

RESS method, along with all the techniques relying on the supercritical fluids, represents the common advantage of not requiring the use of toxic organic solvents and surfactants. This technique can be used, either for size reduction or for encapsulation purposes. A fluid is said supercritical if it's maintained on a temperature and pressure that are higher than those of its critical point. At this state, the fluid possesses very interesting properties for separation and reaction as its density becomes close to a liquid density and as it presents mass transfer properties. Due to these special characteristics, principal parameters tied to the fluid density may be controlled, especially the solvent properties. In fact, variation of the pressure enables the dissolution of molecules when density is close to liquid density and allows the precipitation of the same substances if density is close to a gas density. Carbon dioxide remains by far the most used fluid in supercritical fluid technology because it is nontoxic, non-flammable, and environmentally acceptable. Furthermore, it can be easily transformed to the supercritical state (Yashwant and Deepack, 2009; Davies et al., 2008; Mishima, 2008). In the RESS technique, the drug alone is first dissolved in supercritical CO_2 in high pressure chamber. Subsequently, the solution is passed through a nozzle leading to a rapid decrease of the pressure and consequently a precipitation of the drug particles alone or embedded in the polymer matrix if a polymer is added (Fig. 7). RESS is very interesting for producing submicron particles containing active molecules for drug delivery. Dissolution rates and solubility of a lot of active pharmaceutical ingredients were enhanced. In fact, RESS provided enhancements of dissolution rates of ibuprofen, carbamazepin and naproxen by 6, 2 and 1.2 times, respectively (Bolten and Türk, 2012; Türk and Bolten, 2010). Amorphous films of ketoprofen-PLA were also obtained by RESS (Imran ul-haq et al., 2010).

Hezave and Esmaeilzadeh (2011) investigated the effects of some RESS parameters on the size and the morphology of diclofenac particles. Spraying distance from the tip of the nozzle was among the studied parameters and the obtained results showed an increase of the particles size with an increase of the spraying distance. In addition, an increase of the extraction pressure resulted in a decrease of the particles size. Conversely, an increase of the extraction temperature led to an increase of the particles size. Effect of the nozzle length seems to be conflicting as Wang et al. (2005) and Yildiz et al. (2007) reported an increase in the particle size with an increase of the nozzle length while Kayrak et al. (2003) found opposite results.

Keshavarz et al. (2012) used the RESS technique to prepare nanocrystals of the SERM raloxifene. In fact, diminishing the particle size is one of the adopted approaches for enhancing the bioavailability of active substances through the improvement of their dissolution rate. Authors studied the effect of some RESS process parameters on particles size. An increase in the extraction temperature from 40 to 60 °C led to a decrease in mean particle size from 27.15 to 18.93 nm, but further increase in temperature to 80 °C resulted in an increase in mean particle size. This was explained by the increase of solute's vapor pressure, subsequent to rising

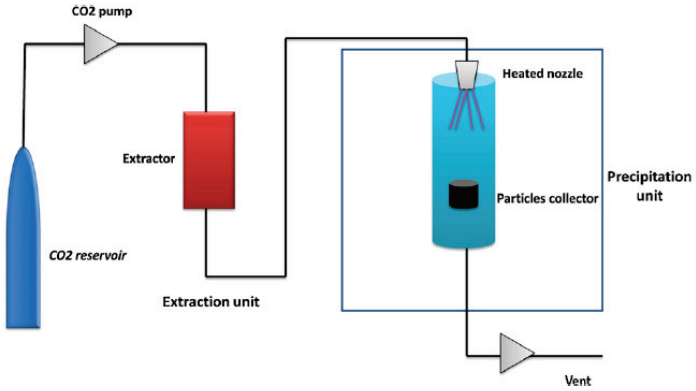


Fig. 7. Rapid expansion of supercritical solutions technique (RESS).

temperature, which increases solubility and thus increases supersaturation. Consequently, reduction in critical nucleus size occurs and therefore, particle size is reduced. However, further increase in temperature increases particle size. In fact, collision is more frequent as a result of excessive increase in number of nuclei because of a higher level of supersaturation.

Extraction pressure also influenced particles size. As pressure increased from 10 to 14 MPa, mean particle size was not significantly reduced but with pressure increase to 18 MPa, mean particle size was reduced to 18.93 nm. Additionally, the increase of spray distance resulted in a decrease of mean particle size. In fact, when spray distance increases particles breakage probability increases and thus, particles become smaller. Moreover, *in vitro* dissolution rate study of raloxifene nanoparticles showed an almost 7-fold increase in dissolution rate compared with the unprocessed form which may increase active bioavailability (Keshavarz et al., 2012).

4.8. Adsorption of antiosteoporotic drugs on particles surface

Drug adsorption may represent an alternative to drug encapsulation when the latter process needs high energy or compromises the stability of the active pharmaceutical ingredient (Alhareth et al., 2010). The adsorption of active molecules can take place on various carriers: liposomes, polymeric biodegradable (biodegradable polyesters based particles) particles or biopersistent (titanium dioxide based) particles (Vrignaud et al., 2011). Adsorption depends on physicochemical properties of the active and on the nature of nanoparticle surface. For instance, electrostatic forces managed the process of adsorption of doxorubicin hydrochloride on poly(butylcyanoacrylate) particles which also implied the crucial effect of the pH of the medium (Yang et al., 2000; Brasseur et al., 1991). An explication for the mechanism of adsorption was proposed by Pitaksuteepong et al. (2002) who mentioned the hypothesis of drug penetration and diffusion within the polymer bulk after they noticed that hydrophilic macromolecules charge had no effect on the amount of adsorbed molecules. Authors also reported that for these same molecules, drug adsorption diminished when molecular weight increased. Practically, drug adsorption is carried by a simple incubation of a drug with particles (Fig. 8). The drug concentration plays an actual role in the resulting loading efficiency.

Table 6 shows different antiosteoporotic drugs loaded by adsorption on nanoparticles surface. Dissette et al. (2010) used the adsorption technique to load RS on titanium dioxide particles by a simple incubation of an aqueous solution of RS in presence of either nanocrystalline or colloidal TiO_2 and adding 0.1 M NaOH to provide a pH of 5.5. Higher drug adsorption was obtained with colloidal TiO_2 (7.2%) compared with 4% for adduct obtained by nanocrystalline TiO_2. *In vivo* studies indicated that the microparticles obtained with colloidal TiO_2 were able to prolong the presence of RS in the bloodstream for 8 h, resulting in a relative bioavailability almost doubled with respect to the free drug.

Li et al. (2010) adsorbed bone modeling protein BMP-2 on gelatin microspheres then added calcium phosphate cement to the formulation. Two formulations were compared in the base of *in vitro* and *in vivo* studies: a blend constructed of BMP-2 loaded microspheres and calcium phosphate cement (BMP-2/GM/CPC) and a BMP-2 and CPC containing blend (BMP-2/CPC). Both formulations were presented as implants. The *in vitro* study showed that the new composite (BMP-2/GM/CPC) released more BMP-2 compared with BMP-2/CPC. Additionally, both composites were implanted in bone defects of osteoporotic goats and left in place for 45 and 140 days. Computed tomography revealed that the bone defects healed more quickly with new grafts. On the other hand, after 45 days of implantation, bone mineralization rate was greater in BMP-2/GM/CPC than in BMP-2/CPC.

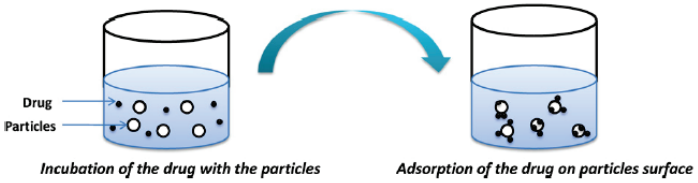


Fig. 8. Adsorption of drug molecules on particles surface.

Table 6
Particulate formulations prepared by the adsorption method.

Pharmaceutical formulation	Polymer or material	API	Particles size	Route	Efficacy study	References
Adducts	TiO_2	Risedronate sodium	211 nm	Oral	*In vitro* *In vivo*	Dissette et al. (2010)
Calcium phosphate cements and microspheres	Gelatin and Calcium phosphate cement	BMP-2	–	Implantation	*In vitro* *In vivo*	Li et al. (2010)
Nanoparticles	Poly-isobutyl-cyanoacrylate	Calcitonin	150 nm	Injectable	*In vitro* *In vivo*	Tasset et al. (1995)
Microspheres	PLGA	Calcitonin	14.8 μm	Intramuscular	–	Calis et al. (1995)

Tasset et al. (1995) used the adsorption method to prepare poly(isobutylcyanoacrylate) nanoparticles loaded with CT. A percentage of binding of CT to the polymer of 96.3% was obtained. This was explained by high affinity between CT and poly(isobutylcyanoacrylate). Authors compared *in vivo* efficiency of different formulations: free CT, internal loading of CT (CT–NP) and the adsorption method on preformed unloaded nanoparticles (CT/NP). After subcutaneous administration, the most important decrease of animals' calcemia was observed for CT/NP with a 28% decrease 6.25 h after the administration. In addition, CT level was higher after CT/NP injection than after CT–NP or free CT injection.

Calis et al. (1995) investigated the adsorption of CT on PLGA microparticles. Obtained data showed that PLGA microparticles have high adsorption capacity for CT which is dependent on polymer and peptide concentrations. Adsorption of CT on microparticles was explained by three phenomena: (a) peptide–polymer interaction followed by adsorption of CT on the polymeric surface, (b) adsorption of peptide on polymer which is followed by the adsorption of another peptide to the previously adsorbed one and (c) the adsorption of associated peptide molecules on polymer surface, which occurred at high peptide concentrations.

5. Concluding remarks

Osteoporosis remains a debilitating disease which affects bone and may lead to high morbidity and mortality. Pharmacological therapy presents some limitations related to bioavailability issues. Therefore, attempts have been made to overcome such shortcomings by formulating various carrier systems. Most common methods used for the preparation of these carriers include emulsion solvent evaporation, spray drying, emulsion polymerization (or polymerization in dispersed media), ionic gelation, film hydration method, emulsion solvent diffusion and adsorption technique. Approaches used for formulation include the use of polymers that allow the mucoadhesion of the drug delivery system to the gastrointestinal wall (BP encapsulated in chitosan derivative polymers). Other methods include the preparation of a sustained release formulation to prolong the half-life of the active substance and thus, promote patient compliance (encapsulation of estradiol in PLGA microspheres). Size reduction of the particles was also an alternative to enhance the dissolution rate of hydrophobic drugs (raloxifene nanoparticles obtained by RESS). Protection of peptidic drugs like CT and PTH from proteolytic activity by encapsulation was also a common option. These different formulations were tested *in vitro* and *in vivo* with variable results depending on the route of administration, the operating conditions and particles constituents and size.

Enhancement of the bioavailability of antiosteoporotic drugs was obtained by encapsulation and adsorption to different polymers. Along these studies, better management of operational conditions and polymer and/or excipients' selection allowed a great improvement of EE. However, further studies should be conducted as the use of these carrier systems still lack in human osteoporosis treatment.

Newer research papers are now focusing on the design of pharmaceutical formulations which specifically target the bone. This approach relies on the use of chemical substances that present affinity to bone tissue such as tetracyclines, BP, and estradiol analogs (Low and Kopecek, 2012; Luhmann et al., 2012; Wang et al., 2012b). Specific targeting may represent an additional interesting alternative for further improvement of the efficiency of osteoporosis treatment.

Acknowledgement

K. Miladi would like to acknowledge support from the Tunisian Ministry of Higher Education and Scientific Research by awarding a grant.

References

Alhareth, K., Vauthier, C., Gueutin, C., Ponchel, G., Moussa, F., 2010. Doxorubicin loading and *in vitro* release from poly(alkylcyanoacrylate) nanoparticles produced by redox radical emulsion polymerization. J. Appl. Polym. Sci. 119, 816–822.
Avadi, M.R., Sadeghi, A.M., Mohammadpour, N., Abedin, S., Atyabi, F., Dinarvand, F., Rafiee-Tehrani, M., 2010. Preparation and characterization of insulin nanoparticles using chitosan and Arabic gum with ionic gelation method. Nanomed. Nanotechnol. Biol. Med. 6, 58–63.
Aydin, Z., Akbuga, J., 1996. Chitosan beads for the delivery of salmon calcitonin: preparation and release characteristics. Int. J. Pharm. 131, 101–103.
Baek, J.S., Kwon, H.H., Hwang, J.S., Sung, H.C., Lee, J.M., Shin, S.C., Kim, Y.R., Cho, C.W., 2011. Alendronate-loaded microparticles for improvement of intestinal cellular absorption. J. Drug Target. 19, 37–48.
Barratt, G., 2003. Colloidal drug carriers: achievements and perspectives. Cell. Mol. Life Sci. 60, 21–37.
Beija, M., Salvayre, R., Lauth-de Viguerie, N., Marty, J.D., 2012. Colloidal systems for drug delivery: from design to therapy. Trends Biotechnol. 30, 485–496.
Bernkop-Schnürch, A., Weithaler, A., Albrecht, K., Greimel, A., 2006. Thiomers: preparation and in vitro evaluation of a mucoadhesive nanoparticulate drug delivery system. Int. J. Pharm. 317, 76–81.
Binkley, N., Bolognese, M., Sidorowicz-Bialynicka, A., Vally, T., Trout, R., Miller, C., Buben, C.E., Gilligan, J.P., Krause, D.S., 2012. A phase 3 trial of the efficacy and safety of oral recombinant calcitonin: the ORACAL trial. J. Bone Miner. Res. 27, 1821–1829.
Boanini, E., Gazzano, M., Rubini, K., Bigi, A., 2007. Composite nanocrystals provide new insight on alendronate interaction with hydroxyapatite structure. Adv. Mater. 19, 2499–2502.
Bolten, D., Türk, M., 2012. Micronization of carbamazepin through rapid expansion of supercritical fluids (RESS). J. Supercrit. Fluids 66, 389–397.
Brar, K.S., 2010. Prevalent and emerging therapies for osteoporosis. Med. J. Armed Forces India 66, 249–254.
Brasseur, N., Brault, D., Couvreur, P., 1991. Adsorption of hematoporphyrin onto polyalkylcyanoacrylate nanoparticles: carrier capacity and drug release. Int. J. Pharm. 70, 129–135.
Calis, S., Jeyanthi, R., Tsai, T., Mehta, R.C., Deluca, P.P., 1995. Adsorption of salmon calcitonin to PLGA microspheres. Pharmaceut. Res. 12, 1072–1076.
Call, K., Sollohub, K., 2010. Spray drying technique. I: hardware and process parameters. J. Pharm. Sci. 99, 575–586.
Cenni, E., Granchi, D., Avnet, S., Fotia, C., Salerno, M., Micieli, D., Sarpietro, M.G., Pignatello, R., Castelli, F., Baldini, N., 2008. Biocompatibility of poly(D,L-lactide-co-glycolide) nanoparticles conjugated with alendronate. Biomaterials 29, 1400–1411.
Chen, Y., Lu, Y., Chen, J., Lai, J., Sun, J., Hu, F., Wu, W., 2009. Enhanced bioavailability of the poorly water-soluble drug fenofibrate by using liposomes containing a bile salt. Int. J. Pharm. 376, 153–160.
Christiansen, C., 1996. Hormone replacement therapy and osteoporosis. Maturitas 23, 71–76.
Cintra e Silva, D.O., Estevanato, L.L., Simioni, A.R., Rodrigues, M.M., Lacava, B.M., Lacava, Z.G., Tedesco, A.C., Morais, P.C., Bào, S.N., 2012. Sucessful strategy for

targeting the central nervous system using magnetic albumin nanospheres. J. Biomed. Nanotechnol. 8, 182–189.
Codrons, V., Vanderbist, F., Verbeeck, R.K., Arras, M., Lison, D., Préat, V., Vanbever, R., 2003. Systemic delivery of parathyroid hormone (1–34) using inhalation dry powders in rats. J. Pharm. Sci. 92, 938–950.
Cremers, S.C., Pillai, G., Papapoulos, S.E., 2005. Pharmacokinetics/pharmacodynamics of bisphosphonates: use for optimisation of intermittent therapy for osteoporosis. Clin. Pharmacokinet. 44, 551–570.
Cruz, L., Assumpção, E., Andrade, S.F., Conrado, D.J., Guterres, S.S., Pohlmann, A.R., 2010a. Microencapsulation of sodium alendronate reduces drug mucosal damage in rats. Drug Deliv. 17, 231–237.
Cruz, L., Assumpção, E., Andrade, S.F., Conrado, D.J., Kulkamp, I.C., Guterres, S.S., Pohlmann, A.R., 2010b. Gastroresistant microparticles containing sodium alendronate prevent the bone loss in ovariectomized rats. Eur. J. Pharm. Sci. 40, 441–447.
Cruz, L., Assumpção, E., Guterres, S.S., Pohlmann, A.R., 2009. High encapsulation efficiency of sodium alendronate in Eudragit S100/HPMC blend microparticles. Quim. Nova 32, 1170–1174.
Cruz, L., Fattal, E., Tasso, L., Freitas, G.C., Carregaro, A.B., Guterres, S.S., Pohlmann, A.R., Tsapis, N., 2011. Formulation and *in vivo* evaluation of sodium alendronate spray-dried microparticles intended for lung delivery. J. Control. Release 152, 370–375.
Davies, O.R., Lewis, A.L., Whitaker, M.J., Tai, H., Shakesheff, K.M., Howdle, S.M., 2008. Applications of supercritical CO_2 in the fabrication of polymer systems for drug delivery and tissue engineering. Adv. Drug Deliv. Rev. 60, 373–387.
Dissette, V., Bozzi, P., Bignozzi, C.A., Dalpiaz, A., Ferraro, L., Beggiato, S., Leo, E., Vighi, E., Pasti, L., 2010. Particulate adducts based on sodium risedronate and titanium dioxide for the bioavailability enhancement of oral administered bisphosphonates. Eur. J. Pharm. Sci. 41, 328–336.
Doustgani, A., Farahani, E.V., Imani, M., Doulabi, A.H., 2012. Dexamethasone sodium phosphate release from chitosan naopartilces prepared by ionic gelation method. J. Colloid Sci. Biotechnol. 1, 42–50.
Fan, W., Yan, W., Xu, Z., Ni, H., 2012. Formation mechanism of monodisperse, low molecular weight chitosan nanoparticles by ionic gelation technique. Colloids and Surfaces B: Biointerfaces 90, 21–27.
Faucheux, C., Verron, E., Soueidan, A., Josse, S., Arshad, M.D., Janvier, P., Pilet, P., Bouler, J.M., Bujoli, B., Guïcheux, J., 2009. Controlled release of bisphosphonate from a calcium phosphate biomaterial inhibits osteoclastic resorption *in vitro*. Journal of Biomedical Materials Research 89, 46–56.
Follin, S.L., Hansen, L.B., 2003. Current approaches to the prevention and treatment of postmenopausal osteoporosis. American Journal of Health-system Pharmacy 60, 883–904.
Gagliardi, M., Bardi, G., Bifone, A., 2012. Polymeric nanocarriers for controlled and enhanced delivery of therapeutic agents to the CNS. Ther. Deliv. 3, 875–887.
Gennari, L., Merlotti, D., Nuti, R., 2009. Perspectives in the treatment and prevention of osteoporosis. Drugs Today 45, 629–647.
Giri, T.K., Choudhary, C., Ajazuddin, Alexander, A., Badwaik, H., Tripathi, D.K., 2012. Prospects of pharmaceuticals and biopharmaceuticals loaded microparticles prepared by double emulsion. Saudi Pharm. J., http://dx.doi.org/10.1016/j.jsps.2012.05.009.
Glowka, E., Sapin-Minet, A., Leroy, P., Lulek, J., Maincent, P., 2010. Preparation and *in vitro–in vivo* evaluation of salmon calcitonin-loaded polymeric nanoparticles. J. Microencapsul. 27, 25–36.
Goldberg, M., Gomez-Orellana, I., 2003. Challenges for the oral delivery of macromolecules. Nat. Rev. Drug Discov. 2, 289–295.
Grabovac, V., Guggi, D., Bernkop-Schnürch, A., 2005. Comparison of the mucoadhesive properties of various polymers. Adv. Drug Deliv. Rev. 57, 1713–1723.
Hariharan, S., Bhardwaj, V., Bala, I., Sitterberg, J., Bakowsky, U., Ravi Kumar, M.N., 2006. Design of estradiol loaded PLGA nanoparticulate formulations: a potential oral delivery system for hormone therapy. Pharm. Res. 23, 184–195.
Heneweer, C., Gendy, S.E., Penate-Medina, E., 2012. Liposomes and inorganic nanoparticles for drug delivery and cancer imaging. Ther. Deliv. 3, 645–656.
Herrero, E.P., Alonso, M.J., Csaba, N., 2012. Polymer-based oral peptide nanomedicines. Ther. Deliv. 3, 657–668.
Hezave, A.Z., Esmaeilzadeh, F., 2011. The effects of RESS parameters on the diclofenac particle size. Adv. Powder Technol. 22, 587–595.
Hochner-Celnikier, D., 1999. Pharmacokinetics of raloxifene and its clinical application. Eur. J. Obstet. Gynecol. Reprod. Biol. 85, 23–29.
Holroyd, C., Cooper, C., Dennison, E., 2008. Epidemiology of osteoporosis. Best Pract. Res. Clin. Endocrinol. Metab. 22, 671–685.
Huang, W., Wang, Y., Ren, L., Du, C., Shi, X., 2009. A novel PHBV/HA microsphere releasing system loaded with alendronate. Mater. Sci. Eng. C 29, 2221–2225.
Imran ul-haq, M., Chasovskikh, E., Signorell, R., 2010. Phase behavior of ketoprofen-poly(lactic acid) drug particles formed by rapid expansion of supercritical solutions. Langmuir 26, 14951–14957.
Jafarinejad, S., Gilani, K., Moazeni, E., Ghazi-Khansari, M., Najafabadi, A.R., Mohajel, N., 2012. Development of chitosan-based nanoparticles for pulmonary delivery of itraconazole as dry powder formulation. Powder Technol. 222, 65–70.
Josse, S., Faucheux, C., Soueidan, A., Grimandi, G., Massiot, D., Alonso, B., Janvier, P., Laïb, S., Gauthier, O., Daculsi, G., Guicheux, J., Bujoli, B., Bouler, J.M., 2004. Chemically modified calcium phosphates as novel materials for bisphosphonate delivery. Adv. Mater. 16, 1423–1427.
Kawashima, Y., Yamamoto, H., Takeuchi, H., Hino, T., Niwa, T., 1998. Properties of a peptide containing DL-lactide:glycolide copolymer nanospheres prepared by novel emulsion solvent diffusion methods. Eur. J. Pharm. Biopharm. 45, 41–48.
Kayrak, D., Akman, U., Hortaçsu, Ö., 2003. Micronization of ibuprofen by RESS. J. Supercrit. Fluids 26, 17–31.
Khachane, P., Date, A.A., Nagarsenker, M.S., 2011. Eudragit EPO nanoparticles: application in improving therapeutic efficacy and reducing ulcerogenicity of meloxicam on oral administration. J. Biomed. Nanotechnol. 7, 590–597.
Keck, C.M., Müller, R.H., 2006. Drug nanocrystals of poorly soluble drugs produced by high pressure homogenisation. Eur. J. Pharm. Biopharm. 62, 3–16.
Keshavarz, A., Karimi-Sabet, J., Fattahi, A., Golzary, A., Rafiee-Tehrani, M., Dorkoosh, F.A., 2012. Preparation and characterization of raloxifene nanoparticles using Rapid Expansion of Supercritical Solution (RESS). J. Supercrit. Fluids 63, 169–179.
Kim, C.W., Yun, Y.P., Lee, H.J., Hwang, Y.S., Kwon, I.K., Lee, S.C., 2010. In situ fabrication of alendronate-loaded calcium phosphate microspheres: controlled release for inhibition of osteoclastogenesis. J. Control. Release 147, 45–53.
Lamprecht, A., Yamamoto, H., Takeuchi, H., Kawashima, Y., 2004. pH-sensitive microsphere delivery increases oral bioavailability of calcitonin. J. Control. Release 98, 1–9.
Laouini, A., Jaafar-Maalej, C., Limayem-Blouza, I., Sfar, S., Charcosset, C., Elaissari, A., 2012. Preparation, characterization and applications of liposomes: state of the art. J. Colloid Sci. Biotechnol. 1, 147–168.
Law, S.L., Shih, C.L., 2001. Characterization of calcitonin-containing liposome formulations for intranasal delivery. J. Microencapsul. 18, 211–221.
Lecart, M.P., Reginster, J.Y., 2011. Current options for the management of postmenopausal osteoporosis. Expert Opin. Pharmacother. 12, 2533–2552.
Levchenko, T., Hartner, W.C., Torchilin, V.P., 2012. Liposomes for cardiovascular targeting. Ther. Deliv. 3, 501–514.
Li, M., Liu, X., Liu, X., Ge, B., 2010. Calcium phosphate cement with BMP-2-loaded gelatin microspheres enhances bone healing in osteoporosis: a pilot study. Clin. Orthop. Relat. Res. 468, 1978–1985.
Li, P., Wang, Y., Peng, Z., She, F., Kong, L., 2011. Development of chitosan nanoparticles as drug delivery systems for 5-fluorouracil and leucovorin blends. Carbohydr. Polym. 85, 698–704.
Lin, J.H., Duggan, D.E., Chen, I.W., Ellsworth, R.L., 1991. Physiological disposition of alendronate, a potent anti-osteolytic bisphosphonate, in laboratory-animals. Drug. Metab. Dispos. 19, 926–932.
Low, S.A., Kopecek, J., 2012. Targeting polymer therapeutics to bone. Adv. Drug Deliv. Rev. 64, 1189–1204.
Lu, T., Ma, Y., Hu, H., Chen, Y., Zhao, W., Chen, T., 2011. Ethinylestradiol liposome preparation and its effects on ovariectomized rats' osteoporosis. Drug Deliv. 18, 468–477.
Luhmann, T., Germershaus, O., Groll, J., Meinel, L., 2012. Bone targeting for the treatment of osteoporosis. J. Control. Release 161, 198–213.
Ma, J., Feng, P., Ye, C., Wang, Y., Fan, Y., 2001. An improved interfacial coacervation technique to fabricate biodegradable nanocapsules of an aqueous peptide solution from polylactide and its block copolymers with poly(ethylene glycol). Colloid Polym. Sci. 279, 387–392.
MacBane, S., 2011. Osteoporosis: a review of current recommendations and emerging treatment options. Formulary 46, 432–446.
Makhlof, A., Werle, M., Tozuka, Y., Takeuchi, H., 2010. Nanoparticles of glycol chitosan and its thiolated derivative significantly improved the pulmonary delivery of calcitonin. Int. J. Pharm. 397, 92–95.
Mazzaferro, S., Bouchemal, K., Maksimenko, A., Skanji, R., Opolon, P., Ponchel, G., 2012. Reduced intestinal toxicity of docetaxel loaded into mucoadhesive nanoparticles, in mouse xenograft model. J. Colloid Sci. Biotechnol. 1, 210–217.
Meure, L.A., Foster, N.R., Dehghani, F., 2008. Conventional and dense gas techniques for the production of liposomes: a review. Pharm. Sci. Technol. 9, 798–809.
Mishima, K., 2008. Biodegradable particle formation for drug and gene delivery using supercritical fluid and dense gas. Adv. Drug Deliv. Rev. 60, 411–432.
Mittal, G., Sahana, D.K., Bhardwaj, V., Ravi Kumar, M.N., 2007. Estradiol loaded PLGA nanoparticles for oral administration: effect of polymer molecular weight and copolymer composition on release behavior *in vitro* and *in vivo*. J. Control. Release 119, 77–85.
Mohammed, A.R., Weston, N., Coombes, A.G., Fitzgerald, M., Perrie, Y., 2004. Liposome formulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability. Int. J. Pharm. 285, 23–34.
Mondal, T., Sunny, M.C., Khastgir, D., Varma, H.K., Ramesh, P., 2012. Poly(l-lactide-co-ε-caprolactone) microspheres laden with bioactive glass-ceramic and alendronate sodium as bone regenerative scaffolds. Mater. Sci. Eng. C 32, 697–706.
Mora-Huertas, C.E., Fessi, H., Elaissari, A., 2011. Influence of process and formulation parameters on the formation of submicron particles by solvent displacement and emulsification–diffusion methods: critical comparison. Adv. Colloid Interface Sci. 163, 90–122.
Mora-Huertas, C.E., Fessi, H., Elaissari, A., 2010. Polymer-based nanocapsules for drug delivery. Int. J. Pharm. 385, 113–142.
Nafea, E.H., El-Massik, M.A., El-Khordagui, L.K., Marei, M.K., Khalfallah, N.M., 2007. Alendronate PLGA microspheres with high loading efficiency for dental applications. J. Microencapsul. 24, 525–538.
Nasr, M.A., Awad, G.A.S., Mansour, S., Taha, I., Al Shamy, A., Mortada, N.D., 2011. Different modalities of NaCl osmogen in biodegradable microspheres for bone deposition of risedronate sodium by alveolar targeting. Eur. J. Pharm. Biopharm. 79, 601–611.
O'Donnell, P.B., McGinity, J.W., 1997. Preparation of microspheres by the solvent evaporation technique. Adv. Drug Deliv. Rev. 28, 25–42.
Ogawa, Y., Yamamoto, M., Okada, H., Yashiki, T., Shimamoto, T., 1988. A new technique to efficiently entrap leuprolide acetate into microcapsules of polylactic acid or copoly(lactic/glycolic) acid. Chem. Pharm. Bull. 36, 1095–1103.

Otsuka, M., Uenodan, H., Matsuda, Y., Mogi, T., Ohshima, H., Makino, K., 2002. Therapeutic effect of *in vivo* sustained estradiol release from poly (lactide-co-glycolide) microspheres on bone mineral density of osteoporosis rat. Biomed. Mater. Eng. 12, 157–167.
Palmieri, G.F., Bonacucina, G., Di Martino, P., Martelli, S., 2001. Spray-drying a method for microparticulate controlled release systems preparation: advantages and limits. I. Water-soluble drugs. Drug. Dev. Ind. Pharm. 27, 195–204.
Park, S.J., Choi, S.G., Devaa, E., Park, J.S., 2011. Encapsulation enhancement and stabilization of insulin in cationic liposomes. Int. J. Pharm. 415, 267–272.
Paudel, A., Worku, Z.A., Meeus, J., Guns, S., Van den Mooter, G., 2012. Manufacturing of solid dispersions of poorly water soluble drugs by spray drying: formulation and process considerations. Int. J. Pharm., http://dx.doi.org/10.1016/j.ijpharm.2012.07.015.
Perugini, P., Genta, I., Conti, B., Modena, T., Pavanetto, F., 2001. Long-term release of clodronate from biodegradable microspheres. AAPS PharmSciTech 2, 6–14.
Perugini, P., Genta, I., Conti, B., Modena, T., Cocchi, D., Zaffe, D., Pavanetto, F., 2003. PLGA microspheres for oral osteopenia treatment: preliminary "*in vitro*"/"*in vivo*" evaluation. Int. J. Pharm. 256, 153–160.
Peter, B., Pioletti, D.P., Laïb, S., Bujoli, B., Pilet, P., Janvier, P., Guicheux, J., Zambelli, P.Y., Bouler, J.M., Gauthier, O., 2005. Calcium phosphate drug delivery system: influence of local zoledronate release on bone implant osteointegration. Bone 36, 52–60.
Pickar, J.H., MacNeil, T., Ohleth, K., 2010. SERMs: progress and future perspectives. Maturitas 67, 129–138.
Pinto Reis, C., Neufeld, R.J., Ribeiro, A.J., Veiga, F., 2006. Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. Nanomed. Nanotechnol. Biol. Med. 2, 8–21.
Pitaksuteepong, T., Davies, N.M., Tucker, I.G., Rades, T., 2002. Factors influencing the entrapment of hydrophilic compounds in nanocapsules prepared by interfacial polymerisation of water-in-oil microemulsions. Eur. J. Pharm. Biopharm. 3, 335–342.
Poletto, F.S., Fiel, L.A., Lopes, M.V., Schaab, G., André, M.O., Guterres, S.S., Rossi-Bergmann, B., Pohlmann, A.R., 2012. Fluorescent-labeled poly-ε-caprolactone lipid–core nanocapsules: synthesis, physicochemical properties and macrophage uptake. J. Coll. Sci. Biotechnol. 1, 89–98.
Quintanar-Guerrero, D., Fessi, H., 1996. Influence of stabilizing agents and preparative variables on the formation of poly(D,L-lactic acid) nanoparticles by an emulsification–diffusion technique. Int. J. Pharm. 143, 133–141.
Raffin, R.P., Lima, A., Lorenzoni, R., Antonow, M.B., Turra, C., Alves, M.P., Fagan, S.B., 2012. Natural lipid nanoparticles containing nimesulide: synthesis, characterization and in vivo antiedematogenic and antinociceptive activities. J. Biomed. Nanotechnol. 8, 309–315.
Rao, J.P., Geckeler, K.E., 2011. Polymer nanoparticles: preparation techniques and size-control parameters. Prog. Polym. Sci. 36, 887–913.
Ribeiro Rattes, A.L., Oliveira, W.P., 2007. Spray drying conditions and encapsulating composition effects on formation and properties of sodium diclofenac microparticles. Powder Technol. 171, 7–14.
Rosca, I.D., Watari, F., Uo, M., 2004. Microparticle formation and its mechanism in single and double emulsion solvent evaporation. J. Control. Release 99, 271–280.
Roush, K., 2011. Prevention and treatment of osteoporosis in postmenopausal women: a review. Am. J. Nurs. 111, 26–35.
Sahoo, S.K., Parveen, S., Penda, J.J., 2007. The present and future of nanotechnology in human health care. Nanomedicine 3, 20–31.
Sakloetsakun, D., Hombach, J.M., Bernkop-Schnürch, A., 2009. *In situ* gelling properties of chitosan–thioglycolic acid conjugate in the presence of oxidizing agents. Biomaterials 30, 6151–6157.
Samdancioglu, S., Calis, S., Sumnu, M., Atilla Hincal, A., 2006. Formulation and *in vitro* evaluation of bisphosphonate loaded microspheres for implantation in osteolysis. Drug Dev. Ind. Pharm. 32, 473–481.
Seville, P.C., Li, H.Y., Learoyd, T.P., 2007. Spray-dried powders for pulmonary drug delivery. Crit. Rev. Ther. Drug Carrier Syst. 24, 307–360.
Shi, X., Wang, Y., Ren, L., Gong, Y., Wang, D.A., 2009a. Enhancing alendronate release from a novel PLGA/hydroxyapatite microspheric system for bone repairing applications. Pharm. Res. 26, 422–430.
Shi, X., Wang, Y., Ren, L., Zhao, N., Gong, Y., Wang, D.A., 2009b. Novel mesoporous silica based antibiotic releasing scaffold for bone repair. Acta Biomater. 5, 1697–1707.
Shu, X.Z., Zhu, K.J., 2002. The influence of multivalent phosphate structure on the properties of ionically cross-linked chitosan films for controlled drug release. Eur. J. Pharm. Biopharm. 54, 235–243.
Sultana, S., Talegaonkar, S., Ali, R., Mittal, G., Ahmad, F.J., Bhatnagar, A., 2012. Inhalation of alendronate nanoparticles as dry powder inhaler for the treatment of osteoporosis. J. Microencapsul. 29, 1–10.
Takeuchi, H., Matsui, Y., Yamamoto, H., Kawashima, Y., 2003. Mucoadhesive properties of carbopol or chitosan-coated liposomes and their effectiveness in the oral administration of calcitonin to rats. J. Control. Release 86, 235–242.
Tammam, S., Mathur, S., Afifi, N., 2012. Preparation and biopharmaceutical evaluation of tacrolimus loaded biodegradable nanoparticles for liver targeting. J. Biomed. Nanotechnol. 8, 439–449.
Tasset, C., Barette, N., Thysman, C., Ketelslegers, J.M., Lemoine, D., Preat, V., 1995. Polyisobutylcyanoacrylate nanoparticles as sustained release system for calcitonin. J. Control. Release 33, 23–30.
Thakkar, H., Nangesh, J., Parmar, M., Patel, D., 2011. Formulation and characterization of lipid-based drug delivery system of raloxifene-microemulsion and self-microemulsifying drug delivery system. J. Pharm. Bioallied Sci. 3, 442–448.
Tolue, S., Moghbeli, M.R., Ghafelebashi, S.M., 2009. Preparation of ASA (acrylonitrile–styrene–acrylate) structural latexes via seeded emulsion polymerization. Eur. Polym. J. 45, 714–720.
Türk, M., Bolten, D., 2010. Formation of submicron poorly water-soluble drugs by rapid expansion of supercritical solution (RESS): results for naproxen. J. Supercrit. Fluids 55, 778–785.
Uchida, T., Yoshida, K., Goto, S., 1996. Preparation and characterization of polylactic acid microspheres containing water-soluble dyes using a novel w/o/w emulsion solvent evaporation method. J. Microencapsul. 13, 219–228.
Umeki, N., Sato, T., Herada, M., Takeda, J., Saito, S., Iwao, Y., Itai, S., 2010. Preparation and evaluation of biodegradable microspheres containing a new potent osteogenic compound and new synthetic polymers for sustained release. Int. J. Pharm. 392, 42–50.
Vanderhoff, J.W., El-Aasser, M.S., Ugelstad, J., 1979. Polymer emulsification process. US Patent 4,177,177.
Vemuri, S., Rhodes, C.T., 1995. Preparation and characterization of liposomes as therapeutic delivery systems: a review. Pharm. Acta Helv. 70, 95–111.
Vrignaud, S., Benoit, J.P., Saulnier, P., 2011. Strategies for the nanoencapsulation of hydrophilic molecules in polymer-based nanoparticles. Biomaterials 32, 8593–8604.
Vural, I., Sarisozen, C., Olmez, S.S., 2011. Chitosan coated furosemide liposomes for improved bioavailability. J. Biomed. Nanotechnol. 7, 426–430.
Wang, J., Chen, J., Yang, Y., 2005. Micronization of titanocene dichloride by rapid expansion of supercritical solution and its ethylene polymerization. J. Supercrit. Fluids 33, 159–172.
Wang, F., Li, J., Wang, C., 2012a. Hydrophilic and fluorescent colloidal nanorods of MWNTs as effective targeted drug carrier. J. Colloid Sci. Biotechnol. 1, 192–200.
Wang, Y., Tran, K.K., Shen, H., Grainger, D.W., 2012b. Selective local delivery of RANK siRNA to bone phagocytes using bone augmentation biomaterials. Biomaterials 33, 8540–8547.
Wang, Y.W., 2007. Fabrication, characterization and long-term in vitro release of hydrophilic drug using PHBV/HA composite microspheres. Mater. Lett. 16, 1071–1076.
Wei, G., Pettway, G.J., McCauley, L.K., Ma, P.X., 2004. The release profiles and bioactivity of parathyroid hormone from poly(lactic-co-glycolic acid) microspheres. Biomaterials 25, 345–352.
Weidenauer, U., Bodmer, D., Kissel, T., 2003. Microencapsulation of hydrophilic drug substances using biodegradable polyesters. Part I: evaluation of different techniques for the encapsulation of Pamidronate di-sodium salt. J. Microencapsul. 20, 509–524.
Wempe, M.F., Wacher, V.J., Ruble, K.M., Ramsey, M.G., Edgar, K.J., Buchanan, N.L., Buchana, C.M., 2008. Pharmacokinetics of raloxifene in male Wistar–Hannover rats: influence of complexation with hydroxybutenyl-beta-cyclodextrin. Int. J. Pharm. 346, 25–37.
Wu, G., Wang, C., Tan, Z., Zhang, H., 2011. Effect of temperature on emulsion polymerization of n-butyl acrylate. Procedia Eng. 18, 353–357.
Yang, Q., Owusu-Ababio, G., 2000. Biodegradable progesterone microsphere delivery system for osteoporosis therapy. Drug Dev. Ind. Pharm. 26, 61–70.
Yang, S.C., Ge, H.X., Hu, Y., Jiang, X.Q., Yang, C.Z., 2000. Doxorubicin-loaded poly(butylcyanoacrylate) nanoparticles produced by emulsifier-free emulsion polymerization. J. Appl. Polym. Sci. 78, 517–526.
Yashwant, P., Deepack, T., 2009. Drug Delivery Nanoparticles Formulation and Characterization. Informa Health Care, New York.
Yildiz, N., Tuna, S., Döker, O., Calimli, A., 2007. Micronization of salicylic acid and taxol (paclitaxel) by rapid expansion of supercritical fluids (RESS). J. Supercrit. Fluids 41, 440–451.
Zaghloul, A.A., Mustafa, F., Siddiqu, A., Khan, M., 2005. Biodegradable microparticulates of beta-estradiol: preparation and in vitro characterization. Drug Dev. Ind. Pharm. 31, 803–811.
Zhao, S.H., Wu, X.T., Guo, W.C., Du, Y.M., Ling, Y., Jin, T., 2010. N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride nanoparticle as a novel delivery system for parathyroid hormone-related protein 1–34. Int. J. Pharm. 393, 268–272.

Partie expérimentale

En se basant sur les connaissances acquises grâce aux études bibliographiques, on propose de préparer des nanoparticules polymériques chargées en alendronate de sodium. L'alendronate de sodium est une molécule active qui appartient à la famille des bisphosphonates. Les bisphosphonates sont indiqués en première intention pour le traitement de l'ostéoporose. Notre étude expérimentale a été divisée en trois parties. Une première étude consiste en la préparation de nanoparticules à base de poly-ε-caprolactone chargées en alendronate de sodium. Il s'agit d'une étude comparative de deux méthodes de préparation à savoir, la nanoprécipitation et l'émulsion double. Une caractérisation des nanoparticules est réalisée. Une étude du profil de libération *in vitro* est aussi menée. La deuxième partie comporte la préparation de nanoparticules à base de chitosane chargées en alendronate de sodium. Cette fois-ci, les nanoparticules sont préparées par la technique de gélification ionique. Cette méthode présente l'avantage de ne pas utiliser des solvants organiques. Comme la première partie, cette étude comprend aussi une étude systématique de développement, une caractérisation ainsi qu'une étude de la libération *in vitro* de la substance active. Les résultats obtenus sont analysés et discutés sur la base des études bibliographiques. La troisième partie comporte une étude *in vivo* chez le rat des nanoparticules à base de chitosane à la recherche d'un bénéfice thérapeutique. On cherche principalement, une amélioration de la tolérance de la substance active et/ou une amélioration de la biodisponibilité.

Encapsulation de l'alendronate de sodium par nanoprécipitation et émulsion double : de la préparation aux études *in vitro*

Publiée *sous forme d'un article scientifique dans*
« Industrial Crops and Products » :
Industrial Crops and Products *2015; 75 : 24-33.*

Ce premier article traite une étude systématique qui est réalisée sur des nanoparticules de poly-ε-caprolactone contenant de l'alendronate de sodium. L'étude comprend une caractérisation des nanoparticules préparées. Cette caractérisation comporte une étude de la morphologie, la taille, le potentiel Zêta, l'efficacité d'encapsulation et le taux de chargement en actif. Plusieurs paramètres opératoires ont été variés pour évaluer leurs effets sur ces propriétés. Enfin, une étude de libération *in vitro* de la substance active est réalisée et le profil cinétique obtenu est analysé.

L'alendronate de sodium est une molécule active indiquée en première intention pour le traitement de l'ostéoporose. Cependant, l'utilisation de cette substance active en clinique présente quelques inconvénients à savoir, une faible biodisponibilité et des effets indésirables d'irritation touchant le tractus gastro-intestinal. L'alendronate est aussi une molécule de nature hydrophile avec une solubilité maximale de 20mg/ml dans l'eau. La nanotechnologie représente une alternative intéressante pour éviter les inconvénients qui sont rencontrés avec les formes pharmaceutiques conventionnelles. Cependant, l'encapsulation des molécules hydrophiles comme l'alendronate de sodium représente un grand défi. En effet, la grande majorité des techniques permet l'encapsulation des molécules hydrophobes. La poly-ε-caprolactone est un polymère biocompatible et biodégradable qui a été largement utilisé pour des applications pharmaceutiques y compris pour l'encapsulation des molécules actives. Dans cette partie, nous avons préparé et caractérisé des nanoparticules polymériques à base de poly-ε-caprolactone. Dans un second temps, nous avons étudié le profil de libération *in vitro* de l'alendronate à partir de ces vecteurs. Deux méthodes de préparation de nanoparticules ont été utilisées : l'émulsion double et la nanoprécipitation. Les particules ont été caractérisées en utilisant la technique de diffusion de la lumière dynamique (taille), la microscopie électronique à transmission (taille et forme) et la calorimétrie différentielle à balayage (état physique de l'actif et du polymère). Une mesure du potentiel Zêta a été aussi réalisée. Le dosage de l'alendronate de sodium par spectrophotométrie ultraviolet a permis de déterminer le pourcentage d'encapsulation et le taux de chargement en actif. Les conditions opératoires ont été maitrisées pour permettre une optimisation des propriétés des nanoparticules obtenues (taille, potentiel zêta et pourcentage d'encapsulation). Les deux techniques ont donné des nanoparticules ayant des tailles comprises entre 200 et 450 nm. L'efficacité d'encapsulation a atteint 34% dans le cas de l'émulsion double et 18% pour la nanoprécipitation. Ceci démontre

que la technique émulsion double a permis une meilleure encapsulation de l'alendronate. Les paramètres expérimentaux qui ont été variés sont : le ratio phase organique/phase aqueuse, le ratio quantité d'actif/quantité de polymère, le poids moléculaire du polymère, la concentration de l'agent stabilisant, la composition de la phase externe et le mode d'évaporation. Après la préparation des particules, l'influence du pH a été aussi étudiée. Pour les deux méthodes, on constate qu'une augmentation de la quantité ou du poids moléculaire du polymère entraine une augmentation de la taille des nanoparticules. Le ratio phase organique/phase aqueuse a aussi exercé un effet significatif sur la taille des nanoparticules. En effet, une augmentation du volume de la phase aqueuse a entrainé une diminution de la taille des nanoparticules. Les images obtenues par microscopie électronique à transmission ont montré des nanoparticules sphériques présentant une forme régulière. La taille des nanoparticules était comprise entre 195 et 447 nm pour la nanoprécipitation et entre 211 et 445 nm pour l'émulsion double. Le potentiel Zêta a varié entre -0.52 et -10.4 mV. La poly-ε-caprolactone est à l'origine de ces valeurs négatives vu la présence des groupements carboxyles (-COOH) en bout de chaine. Le ratio actif/polymère et le poids moléculaire du polymère n'ont pas significativement influé le potentiel zêta. Par exemple, le potentiel zêta a légèrement diminué de -1.63 mV (préparation DE5) à -3.23 mV (préparation DE8) quand le ratio actif/polymère a diminué de 1/1 to 1/10. Une augmentation de la concentration du stabilisant a entrainé une diminution de la taille des nanoparticules. Pour la nanoprécipitation, une augmentation du volume de la phase aqueuse et donc une diminution du ratio phase aqueuse/phase organique a entrainé une diminution significative de la taille des particules. Ceci peut être expliqué par une diffusion plus rapide du solvant organique qui résulte en une précipitation plus rapide du polymère. Par conséquent, une nucléation plus rapide aura lieu ce qui implique la formation de nanoparticules plus petites. L'augmentation du volume de la phase aqueuse s'est traduite par une diminution du pourcentage d'encapsulation. Ceci peut être expliqué par une diminution de la concentration de l'alendronate ce qui le rend moins accessible à l'encapsulation. Pour les deux techniques, nous avons constaté qu'une augmentation de la quantité du polymère (une diminution du ratio actif/polymère) a entrainé une augmentation significative de la taille et du pourcentage d'encapsulation. Cet effet sur la taille peut être expliqué par une augmentation de la viscosité du solvant organique suite à l'augmentation de la quantité du polymère, ce qui a engendré la formation de particules plus grosses. Des quantités plus importantes de polymère peuvent aussi être à l'origine d'une fréquence de collision élevée entre gouttelettes, ce qui entraine une fusion des particules et finalement, l'obtention de particules de plus grande taille. L'augmentation du pourcentage d'encapsulation qui est obtenue suite à une augmentation de

la quantité du polymère est due à une résistance à la diffusion de la molécule active vers la phase externe. De même, une augmentation de la masse molaire du polymère a résulté en une augmentation significative de la taille des particules et du pourcentage d'encapsulation. L'effet sur l'efficacité d'encapsulation peut être expliqué par une augmentation de la viscosité de la phase organique ce qui a rendu la diffusion de la substance active vers la phase externe plus difficile. Ceci a donné aussi naissance à de plus grandes particules qui pourraient encapsuler plus d'actif. Une augmentation de la quantité du stabilisant a diminué d'une manière non significative la taille des particules. Cet effet peut être expliqué par une augmentation de la viscosité de la phase aqueuse suite à l'augmentation de la quantité d'alcool polyvinylique utilisé comme agent stabilisant. Une augmentation de la quantité d'alcool polyvinylique a augmenté le pourcentage d'encapsulation mais pas pour toutes les formules. Ceci peut être du à la présence d'une plus grande quantité d'alcool polyvinylique à la surface des particules, ce qui peut empêcher la diffusion de la substance active vers la phase externe. Une augmentation de la concentration d'alcool polyvinylique s'est traduite par une augmentation du potentiel Zêta. Ceci peut s'expliquer par l'adsorption du stabilisant à la surface des particules ce qui donné une diminution des charges positives fournies par la poly-ε-caprolactone. L'influence de la composition de la phase externe a été évaluée pour la technique d'émulsion double. Il s'avère qu'une augmentation de la concentration de NaCl dans phase aqueuse externe à 1% a légèrement diminué la taille des particules. En même temps, on constate une légère augmentation du pourcentage d'encapsulation. La diminution de la taille des particules peut être attribuée à une diffusion de l'eau qui suit le gradient osmotique, c'est-à-dire, vers la phase aqueuse externe. En revanche, l'amélioration de l'encapsulation peut être expliquée par l'action osmogène exercée par NaCl qui fournit une barrière contre la diffusion de l'alendronate. Par contre, lorsque la concentration du NaCl a été augmentée au-delà de 1,5%, la taille a augmenté significativement alors que le pourcentage d'encapsulation a diminué. Ceci peut être expliqué par une perturbation des forces ioniques du milieu sous l'action de l'électrolyte ce qui a entrainé une agrégation des particules et par conséquent l'obtention de particules plus grosses. L'évaporation du solvant organique à température ambiante a entrainé une augmentation de la taille des particules. La diminution de la taille des particules, qui a été obtenue dans le cas de l'évaporation sous vide, est due à une nucléation plus rapide des gouttelettes de la phase organique alors que l'amélioration d'encapsulation est due à une plus faible diffusion de l'actif vers la phase externe. Les analyses en calorimétrie différentielle à balayage ont été réalisées pour définir l'état physique de l'actif et du polymère au sein des particules. Les résultats obtenus laissent

suggérer que l'alendronate se trouve dans un état amorphe dans les nanoparticules et qu'il est dispersé d'une manière homogène au sein de la matrice du polymère. Cependant, dans notre cas, une telle conclusion semble être peu probable vu la nature hydrophile de l'alendronate et la nature hydrophobe de la poly-ε-caprolactone. L'alendronate sera plutôt emprisonné à l'intérieur des particules ou bien adsorbé à la surface. L'étude du profil de libération *in vitro* a montré que les nanoparticules préparées ont permis une libération prolongée de l'alendronate de sodium. A des quantités égales et à des poids moléculaires de polymères similaires, la libération de l'alendronate a été plus rapide à partir des nanoparticules préparées par nanoprécipitation. Cependant, le profil de libération *in vitro* à partir de toutes les préparations a été similaire avec une première phase de libération rapide suivie d'une phase plus lente. La première phase rapide serait due à la libération de l'alendronate qui se trouve adsorbé sur la surface des particules. L'analyse des résultats de libération *in vitro* par modélisation mathématique a permis de conclure que la libération de l'alendronate est le résultat de l'association de deux mécanismes : la diffusion de la substance active et le relâchement des chaines du polymère. D'autres études sont nécessaires pour optimiser davantage l'encapsulation et pour obtenir une libération plus rapide de l'actif.

Encapsulation of alendronate sodium by nanoprecipitation and double emulsion: From preparation to *in vitro* studies

K. Miladi[a,b], S. Sfar[b], H. Fessi[a]*, A. Elaissari[a]

[a] University of Lyon, F- 69622, Lyon, France; Lyon 1 University, Villeurbanne, CNRS, UMR 5007, Laboratoire d'Automatique et de Génie des Procédés, LAGEP-CPE-308G, 43 bd. du 11 Nov.1918, F-69622, Villeurbanne, France.
[b] University of Monastir, Laboratoire de pharmacie galénique, Rue Avicenne, 5000 Monastir, Tunisia

* Corresponding author: Phone: +33-472431841, Fax: +33-472431682

E-mail: fessi@lagep.univ-lyon1.fr

Abstract:

Alendronate sodium is an active molecule indicated as first line regimen for osteoporosis treatment. Use of this drug presents, however, some shortcomings such as low oral bioavailability and gastrointestinal mucosa irritation. Nanotechnology remains an attractive approach to overcome such limitations of conventional pharmaceutical forms. However, encapsulation of hydrophilic molecules like alendronate represents a real challenge. Preparation, characterization and drug release behavior of polycaprolactone based nanoparticles loaded with alendronate sodium were investigated in this work. Two strategies were used to prepare these nanocarriers: double emulsion evaporation and nanoprecipitation. Operating conditions were monitored to produce biocompatible nanoparticles with good properties. Both techniques gave nanoparticles with mean diameter in the range of 200-450 nm. Encapsulation efficiency of selected formulae reached 34% for double emulsion and 18% for nanoprecipitation, respectively. Highest drug loading was 20.7% for double emulsion and 8.2% for nanoprecipitation, respectively. For both methods, an increase of polymer molecular weight and polymer amount led to an increase of particle size. For nanoprecipitation, oil to water phase exerted also a significant effect on particle size. The increase of stabilizer amount or the use of rotavopration rather than continuous stirring at ambient temperature decreased particles size. Prepared nanoparticles exhibited sustained release of alendronate. When the same polymer amount and molecular weight was used, drug release of the selected formulae was faster for nanoparticles formulated by the nanoprecipitation method. However, release profiles were similar for both techniques with a first rapid release phase followed by a more

prolonged phase. Analysis of *in vitro* release data showed also that active release mechanism relied on a combination of drug diffusion and polymer relaxation phenomena.

Key words:
Alendronate sodium, double emulsion evaporation, *in vitro*, nanoparticles, nanoprecipitation, osteoporosis, polycaprolactone.

1. Introduction:

Osteoporosis is the most frequent metabolic disease that affects the bone. It is characterized by a low bone mass due to a microarchitectural deterioration of bone tissue. This results in an enhanced bone fragility and fracture risk, particularly for the long bones and the vertebrae. All the osteoporotic fractures are linked with high morbidity. Furthermore, fractures that affect the hip or the vertebrae are associated with high mortality. This makes osteoporosis an actual and serious health problem. Among all the therapeutic approaches intended to manage the disease, bisphosphonates are used as first-line regimen despite their low oral bioavailability (less than 1 % in most cases) and their numerous side effects. The latter are, especially, related to the gastrointestinal tract such as ulcers, esophageal irritations, diarrhea or constipation (Cremers et al. 2005). For example, alendronate sodium (ALD), which is a bisphosphonate, presents an oral bioavailability as low as 0.7%. In fact, it belongs to the fourth class of Biopharmaceutics Classification System because of its poor solubility and low bioavailability (Fasinu et al. 2011). Some approaches have been presented to circumvent these shortcomings, especially, for chronic patients. Among them, for instance, the use of a tablet form containing the more soluble salt alendronate sodium rather than the insoluble acid form alendronic acid (Fosamax®). Some formulations were administered by other routes such as the parenteral or the pulmonary route in order to enhance bioavailability. However, the chronic feature of the disease makes the oral route more comfortable for patients. Encapsulation was shown to enhance therapy efficiency and tolerance. Actually, particulate carrier systems have several advantages over a simple solution or a powder form of drugs as they can (1) insure controlled delivery of the drug; (2) protect the human body from the toxic effects of drugs; (3) enhance the stability and the bioavailability of active substances and (4) mask their unpleasant taste if it exists (Campos et al. 2013; Grando et al. 2013; de Melo et al. 2013; Mazzaferro et al. 2012; Rosset et al. 2012; Lira et al. 2013; Wang et al. 2012). Encapsulation of ALD would be an interesting alternative to enhance its bioavailability and to

protect the gastrointestinal mucosa from local irritation. Polycaprolactone (PCL) is a biocompatible and biodegradable polymer that has been widely used for drug delivery purposes. Furthermore, it has been successfully used for encapsulation to enhance bioavailability of many actives, for targeting and for sustained delivery purposes (Dash and Konkimalla 2012). Double emulsion and nanoprecipitation have been used to prepare nanoparticles loaded with hydrophilic molecules. However, nanoparticles have not been commonly developed for ALD intended to osteoporosis treatment. The aim of this work is to prepare PCL nanoparticles loaded with ALD by double emulsion and nanoprecipitation in order to compare these two methods and to assess the influence of different operational parameters on particles characteristics (Size, charge, encapsulation efficiency, drug loading and *in vitro* drug release). It has to be known that the word "nanoparticles" is used to identify particles that have a diameter that does not exceed 100nm. We will, however, use this term throughout this paper as it is widely used in literature to designate submicron particles in general.

2. *Materials and methods:*

2.1. Materials:

ALD ((4-amino-1-hydroxybutylidene) diphosphonate trihydrate) was purchased from Polpharma, Poland. PCL with weight average molar mass of 14 000, 65 000 and 80 000 g/mol, Mowiol® 4-88 : polyvinylalcohol (PVA) with a molecular weight of 31000 g/mol, Dichloromethane, copper sulfate and sodium chloride were obtained from Sigma Aldrich, Germany. Nitric acid 68 % was obtained from VWR, France. Acetone was obtained from Laurylab, France. All the other reagents were of pharmaceutical grade and were used without further purification.

2.2. Methods:

2.2.1. Preparation of nanoparticles by double emulsion:

One milliliter of a 20 mg/ml aqueous solution of ALD (W1) was emulsified in 10 milliliters of dichloromethane containing PCL. Various amounts and molecular weights of PCL were dissolved. PCL amounts were used to provide drug to polymer rations of 1:1, 1:2, 1:5 and 1/10. Three PCL molecular weights were used 14000, 65000 and 80000 g/mol. Dispersion of the aqueous phase in the organic one was performed by the use of the homogenizer Ultraturrax T25 under 21500 rotations per minute (rpm) to obtain the first simple emulsion

(W1/O). The latter was dispersed by an Ultraturrax T25 under 21500 rpm in 0.5% (w/v) PVA aqueous solution (W2) which led to the formation of a double emulsion (W1/O/W2). Both dispersions were carried out during 15 minutes in an ice bath. Then, solvent evaporation was carried out by Büchi Rotavapor R-124 under high temperature and reduced pressure conditions. These experiments were performed to assess the influence of evaporation method on particles. Table 1 summarizes the different formulations prepared by double emulsion. The suspension of nanoparticles was subjected to ultracentrifugation at 30 000 rpm during 30 minutes by an ultracentrifuge Optima (Beckman Coulter, France). The supernatant and the sediment were both collected.

Table1. Composition of formulations prepared by the double emulsion

Formulation code	Polymer	Ratio drug to polymer	NaCl (%)
DE1	*PCL 14000*	*1:1*	-
DE2		*1:2*	-
DE3		*1:5*	-
DE4		*1:10*	-
DE5	*PCL 65000*	*1:1*	-
DE6		*1:2*	-
DE7		*1:5*	-
DE8		*1:10*	-
DE9	*PCL 80000*	*1:1*	-
DE10		*1:2*	-
DE11		*1:5*	-
DE12		*1:10*	-
DE13	*PCL 80000*	*1:10*	*0.5*
DE14		*1:10*	*1*
DE15		*1:10*	*1.5*
DE16		*1:10*	*2*

2.2.2. Preparation of nanoparticles by nanoprecipitation:

The nanoparticles were prepared by nanoprecipitation method as described by (Fessi et al. 1989). Different amounts and various molecular weights of PCL were dissolved in 10 ml of acetone under mild heating. This organic phase was added drop wise to various volumes of aqueous solution containing 0.5% (w/v) PVA and 20 milligrams of ALD. PCL amounts were used to provide drug to polymer rations of 1:1, 1:2, 1:5 and 1:10. Three PCL molecular weights were used 14000, 65000 and 80000 g/mol. The obtained suspension was stirred magnetically for 10 minutes. Solvent evaporation was carried subsequently by Büchi Rotavapor R-124 (under high temperature and reduced pressure conditions). The obtained

suspension was subjected to ultracentrifugation at 30 000 rpm for 30 minutes by an ultracentrifuge Optima (Beckman Coulter, France). The supernatant was collected and the precipitated nanoparticles were recovered and washed by a 0.9% NaCl solution to undergo ALD determination and release studies. Table 2 summarizes the compositions of the different formulations prepared by nanoprecipitation.

2.2.3. Characterization of the prepared nanoparticles:

2.2.3.1. Particle size, size distribution and Zeta potential:

Z-average diameter of the prepared nanoparticles and their Zeta potential were determined using Malvern particle size analyzer (Model-Nano ZS, Malvern Instruments limited, UK). The nanoparticles were dispersed in a 1 mM NaCl before each measure. All measurements were carried out in triplicate at 25°C.

2.2.3.2. pH influence on the zeta potential of the nanoparticles:

Studies on influence of pH on zeta potential were carried out for 3 selected formulae from each method: DE4, DE8 and DE14 for double emulsion and NP4, NP16 and NP28 for nanoprecipitation. Initial pH of the obtained nanoparticles suspension was measured by pH meter SensION+ PH3 (HACH, USA). Suspensions were then subjected to pH change to values of 2, 4, 6, 8, 10 and 12. Measures were performed using the same apparatus described above used for the determination of particles size and zeta potential.

2.2.3.3 Morphology and surface characteristics:

Nanoparticles shape and appearance were examined by transmission electron microscopy (TEM) apparatus Philips CM-120 at an accelerating voltage of 100 kV. A drop of the nanoparticles' suspension was withdrawn with a micropipette then placed on a carbon-coated copper grid. The excess of the suspension was removed by blotting the grid with a filter paper. Then the deposit was left to dry before analysis.

Table2. Composition of formulations obtained by nanoprecipitation

Formulation code	Polymer	Ratio drug to polymer	Ratio oil to water phase
NP1	*PCL 14000*	*1:1*	*1:2.5*
NP2		*1:2*	
NP3		*1:5*	
NP4		*1:10*	
NP5		*1:1*	*1:3*
NP6		*1:2*	
NP7		*1:5*	
NP8		*1:10*	
NP9		*1:1*	*1:3.5*
NP10		*1:2*	
NP11		*1:5*	
NP12		*1:10*	
NP13	*PCL 65000*	*1:1*	*1:2.5*
NP14		*1:2*	
NP15		*1:5*	
NP16		*1:10*	
NP17		*1:1*	*1:3*
NP18		*1:2*	
NP19		*1:5*	
NP20		*1:10*	
NP21		*1:1*	*1:3.5*
NP22		*1:2*	
NP23		*1:5*	
NP24		*1:10*	
NP25	*PCL 80000*	*1:1*	*1:2.5*
NP26		*1:2*	
NP27		*1:5*	
NP28		*1:10*	
NP29		*1:1*	*1:3*
NP30		*1:2*	
NP31		*1:5*	
NP32		*1:10*	
NP33		*1:1*	*1:3.5*
NP34		*1:2*	
NP35		*1:5*	
NP36		*1:10*	

2.2.3.4. Differential Scanning Calorimetry (DSC) analysis:

The supernatant which was obtained after ultracentrifugation was left to dry in a hod. Then, nanoparticles were subjected to thermal analysis in order to assess the physical state of the polymer and the drug in the formulations and to study the interactions between them. Experiments were performed on drug alone, drug-free nanoparticles and drug-loaded

nanoparticles. DSC scan of ALD crystals was used as a control. The used apparatus was Differential Scanning Calorimeter Q200 (TA instruments, USA). The thermal behavior was studied by heating approximately 10 mg (±2.0 mg) of the samples in a covered aluminum sample pan under dry nitrogen atmosphere. Temperature range used was between 20 and 160°C with a heating rate of 10°C/min.

2.2.3.5. Encapsulation efficiency and drug loading:

The amount of ALD loaded in nanoparticles was determined by ultraviolet spectrophotometer UV-1800 (Shimadzu, Japan) by the indirect method. The obtained particles were subjected to ultracentrifugation at a speed of 30000 rpm during 30 minutes. The amount of ALD was then determined in the supernatant using the method developed by (Ostović et al. 1993) and previously used by (Cohen-Sela et al. 2006). Amount was determined at 240 nm wavelength following the addition of copper (II) reagent (5 mM copper sulfate in $1.5x10^{-3}M$ HNO_3) that could form a complex between ALD and copper ions. Encapsulated drug amount was obtained by subtraction the amount of active molecule in the supernatant from the initial amount of ALD. Drug loading was expressed as the encapsulated drug amount to the polymer quantity ratio. Encapsulation efficiency was, however, expressed as the encapsulated drug the amount to the initial drug amount. Drug loading and encapsulation efficiency were calculated according to the following equations:

$$\text{Drug loading} = \frac{Amount\ of\ encapsulated\ drug}{Amount\ of\ polymer}\ x\ 100$$

$$\text{Encapsulation efficiency} = \frac{Amount\ of\ encapsulated\ drug}{Initial\ quantity\ of\ drug\ used\ in\ formula}\ x\ 100$$

2.2.3.6. Influence of evaporation method and stabilizer amount:

This influence was assessed for 3 selected formulae from each method: DE4, DE8 and DE14 for double emulsion and NP4, NP16 and NP28 for nanoprecipitation. Organic solvent evaporation was carried out either by rotavapor (Büchi Rotavapor R-124) or by continuously stirring nanoparticles suspension at room temperature for 4 hours. Three surfactant concentrations were used 0.25%, 0.5% and 1%. Influence of these parameters on particle size and encapsulation efficiency was evaluated.

2.2.4. In vitro release studies:

In vitro release of ALD from nanoparticles was investigated for three selected formulations from each method (NP4, NP16 and NP28 for nanoprecipitation and DE4, DE8 and DE14 for double emulsion). Studies were performed in a dissolution apparatus using the basket method at a rotation speed of 100 rpm. Nanoparticles were dispersed in 900 ml of phosphate buffer pH6.8 at 37°C. At defined intervals, 10 milliliters of the release medium were recovered and replaced by 10 milliliters of fresh medium to maintain sink conditions. The released drug amount was measured by UV spectrophotometry by using copper (II) reagent described above. Each sample was immediately centrifuged at 30000 rpm for 15 minutes to separate particles and released drug amount was measured in supernatant. Measurements were carried out in triplicate and obtained data were plotted against time. Separated nanoparticles were immediately re-dispersed in 10 milliliters of fresh phosphate buffer pH6.8 and transferred into the vessel. Release kinetics were also analyzed and fitted to different release models.

2.2.5. Statistical Analysis

All data in tables and figures were expressed as mean ± standard deviation. Statistical analysis was carried out using the one-way analysis of variance (ANOVA) and Student's t tests. $p<0.05$ was chosen as criterion for statistical significance.

3. Results and discussion:

ALD is a hydrophilic molecule which is soluble in water and practically insoluble in most of the organic solvents such as alcohols and methylene chloride (Sweetman 2009). In this work, we assessed two preparation methods to encapsulate ALD: double emulsion and nanoprecipitation. The latter has rarely been used to encapsulate hydrophilic molecules due to its low potency to bear nanoparticles with good encapsulation efficiency but some data proved that hydrophilic molecules have been successfully encapsulated by the technique (Bilati et al. 2005). Hydrophilic active could then be dissolved in the aqueous phase or a cosolvent could be added to allow drug dissolution in the organic phase. Another approach is to transform the hydrophilic molecule to a hydrophobic one before encapsulating it (by esterification, acidification, or alcanization) which permits subsequently its dissolution in an organic solvent. In our case, such a strategy was not possible because ALD possesses multiple pKa (0.8, 2.2, 6.3, 10.9 and 12.2) and it is insoluble in most of the organic solvents either in its

acidic or alkaline form (Ezra and Golomb 2000). This led to many attempts to use the technique to prepare carriers loaded with hydrophilic molecules and encouraged us to assess the applicability of nanoprecipitation for the encapsulation of ALD. Solvent evaporation was the first method developed to prepare polymer nanoparticles from a preformed polymer by (El-Aasser et al. 1979). Double emulsion technique is, specifically, used for the loading of hydrophilic molecules (de Araújo et al. 2013). However, the tendency of the drug to diffuse to the outer phase is a real concern. Several strategies could be used to limit this phenomenon: (1) addition of a salt; (2) addition of a viscosity increasing agent and (3) increase of drug-polymer interaction.

3.1. Characterization of the prepared nanoparticles:

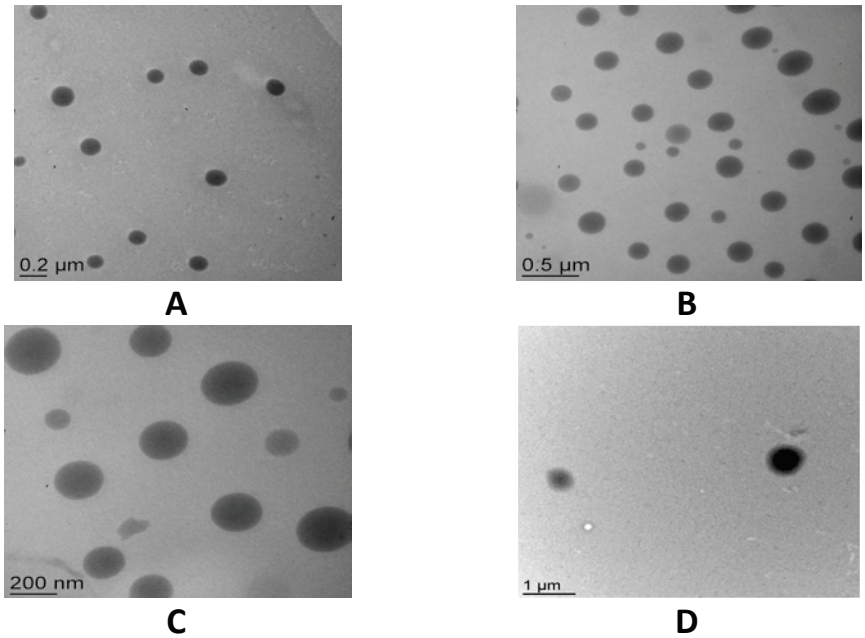


Fig. 1. TEM images of selected formulae (A: DE1; B: DE4; C: NP8; D: NP28)

Table 3 and Table 4 show particle size, zeta potential, encapsulation efficiency and drug loading of prepared particles by both techniques. TEM images showed nanoparticles with a spherical and regular form (See Fig. 1). Nanoparticles size was in the range 195-447 nm for nanoprecipitation and in the range 211-445 nm for double emulsion. Therefore, preparation technique did not influence significantly size range. Zeta potential ranged from -0.52 to -10.4 mV depending on formulations. PCL exhibited a negative value of zeta potential due to the superficial availability of COOH groups (Seremeta et al. 2013). Drug to polymer ratio and polymer molecular weight did not significantly influence zeta potential. For instance, zeta potential decreased slightly from -1.63 mV (formulation DE5) to -3.23 mV (Formulation

DE8) when drug to polymer ratio increased from 1:1 to 1:10. Zeta potential value increased from -3.45 mV (formulation DE1) to -0.57 mV (formulation DE9) when polymer molecular weight increased from 14000 g/mol to 80000 g/mol. As a rule of thumb, absolute zeta potential values above 60 mV yields excellent stability, while absolute values above 30, 20 and less than 5 mV generally results in good stability, acceptable short-term stability and fast particle aggregation, respectively (Riddick and inc 1968). However, this rule is valid only for low-molecular weight surfactants and pure electric stabilization, but not for higher or large molecular weight stabilizers which act mainly by steric stabilization such as, PVA (Lindfors et al. 2006). In this case, even small zeta potential values could ensure sufficient stabilization. In fact, adsorbed layers of polymers/large molecules shift the plane of shear to a farer distance from the particle surface which leads to a decrease of the measured zeta potential. Therefore, despite the low measured zeta potential, nanoparticles suspensions are stable in this case (Mishra et al. 2009). In fact, selected formulations (DE4, DE8, DE14, NP4, NP16, and NOP28) were stable and no aggregation was noticed after storage for 3 months at ambient temperature.

Encapsulation efficiency ranged from 15.12% to 34.31% for double emulsion and from 0.36% to 18.8% for nanoprecipitation. Consequently, double emulsion provided higher encapsulation efficiency than nanoprecipitation. Poor encapsulation could be attributed to poor interaction between hydrophobic PCL and hydrophilic ALD. Double emulsion provided the highest encapsulation efficiency (34.31% for formulation DE14). Likely, drug loading as high as 20.63% was obtained by this technique. This value is higher than that obtained by (Cohen-Sela et al. 2006) (12.24%) and slightly higher than the value obtained by (Cohen-Sela et al. 2009) (19.42%). The highest values for encapsulation efficiency and drug loading obtained by nanoprecipitation technique were 18.8% and 9.16%, respectively. Obviously, double emulsion ensured better encapsulation of ALD. This finding is consistent with other reported data in literature (Han et al. 2013). Consequently, water-in-oil-in-water (w/o/w) emulsification process is the method of choice for the encapsulation inside polymeric particles of hydrophilic drugs having either low or high molecular weight. Conversely, nanoprecipitation is more suitable for the encapsulation of hydrophobic molecules that could be easily dissolved in the inner organic phase.

Table 3. Characteristics of nanoparticles prepared by double emulsion

Formulation code	Z-average diameter (nm)	Zeta potential (mV)	EE (%)	DL (%)
DE1	*211*	*-3.45±0.13*	*15.12±0.61*	*15.12±0.61*
DE2	*225*	*-6.29±0.09*	*17.61±0.44*	*8.8±0.22*
DE3	*245*	*-7.87±0.1*	*19.64±0.56*	*3.93±0.11*
DE4	*272*	*-10.4±0.1*	*23.76±0.52*	*2.38±0.05*
DE5	*282*	*-1.63±0.04*	*19.00±0.72*	*19.00±0.72*
DE6	*294*	*-2.23±0.12*	*21.09±0.5*	*10.54±0.25*
DE7	*300*	*-2.58±0.11*	*23.99±0.61*	*4.8±0.12*
DE8	*316*	*-3.23±0.07*	*27.12±1.12*	*2.71±0.11*
DE9	*336*	*-0.57±0.09*	*20.68±0.66*	*20.68±0.66*
DE10	*348*	*-0.73±0.05*	*25.79±0.66*	*12.9±0.33*
DE11	*357*	*-0.82±0.04*	*27.87±0.78*	*5.57±0.16*
DE12	*368*	*-1.27±0.14*	*31.06±0.63*	*3.06±0.06*
DE13	*351*	*-1.61±0.04*	*32.1±0.46*	*3.21±0.05*
DE14	*339*	*-1.19±0.2*	*34.31±0.96*	*3.43±0.1*
DE15	*392*	*-2.14±0.18*	*30.42±0.61*	*3.04±0.06*
DE16	*430*	*-1.17±0.11*	*25.44±0.72*	*2.54±0.07*

Table 4. Characteristics of nanoparticles prepared by nanoprecipitation

Formulation code	Z-average diameter (nm)	Zeta potential (mV)	EE(%)	DL(%)
NP1	*308*	*-3.07±0.06*	*2.09±0.27*	*2.09±0.27*
NP2	*320*	*-3.6±0.07*	*4.52±0.29*	*2.26±0.15*
NP3	*356*	*-6.67±0.28*	*8.4±0.35*	*1.68±0.07*
NP4	*360*	*-8.25±0.45*	*9.59±0.27*	*0.96±0.03*
NP5	*244*	*-3.07±0.06*	*0.36±0.25*	*0.36±0.25*
NP6	*253*	*-3.23±0.07*	*2.49±0.41*	*1.24±0.2*
NP7	*277*	*-6.9±0.08*	*5.54±0.72*	*1.11±0.14*
NP8	*295*	*-7.57±0.21*	*6.37±0.43*	*0.64±0.04*
NP9	*195*	*-2.12±0.19*	*0.75±0.35*	*0.75±0.35*
NP10	*216*	*-2.28±0.06*	*1.55±0.33*	*0.78±0.17*
NP11	*227*	*-3.41±0.2*	*3.14±0.45*	*0.63±0.09*
NP12	*238*	*-6.82±0.16*	*6.01±0.17*	*0.60±0.02*
NP13	*325*	*-1.13±0.09*	*8.22±0.29*	*8.22±0.29*
NP14	*341*	*-1.57±0.1*	*8.76±0.22*	*4.38±0.11*
NP15	*350*	*-1.72±0.34*	*10.21±0.33*	*2.04±0.07*
NP16	*372*	*-2.13±0.05*	*12.78±0.19*	*1.28±0.02*
NP17	*271*	*-1.04±0.02*	*6.7±0.29*	*6.7±0.29*
NP18	*290*	*-1.56±0.08*	*6.48±0.22*	*3.24±0.11*
NP19	*305*	*-1.64±0.28*	*7.86±0.35*	*1.57±0.07*
NP20	*343*	*-2.02±0.14*	*9.96±0.71*	*1.00±0.07*
NP21	*230*	*-0.78±0.05*	*2.24±0.54*	*2.24±0.54*
NP22	*244*	*-0.99±0.05*	*3.58±0.49*	*1.79±0.25*
NP23	*265*	*-1.09±0.06*	*4.78±0.44*	*0.96±0.09*
NP24	*271*	*-1.18±0.11*	*5.17±0.22*	*0.52±0.02*
NP25	*351*	*-0.66±0.03*	*9.16±0.45*	*9.16±0.45*
NP26	*367*	*-0.68±0.03*	*10.21±0.45*	*5.11±0.23*
NP27	*411*	*-0.84±0.1*	*11.99±0.60*	*2.40±0.12*
NP28	*447*	*-1.03±0.03*	*18.80±0.70*	*1.88±0.07*
NP29	*309*	*-0.69±0.08*	*4.88±0.35*	*4.88±0.35*
NP30	*341*	*-0.72±0.04*	*5.75±0.60*	*2.88±0.3*
NP31	*362*	*-0.72±0.01*	*7.89±0.50*	*1.58±0.1*
NP32	*405*	*-0.85±0.06*	*10.03±0.33*	*1.00±0.03*
NP33	*280*	*-0.53±0.02*	*4.20±0.54*	*4.20±0.54*
NP34	*305*	*-0.57±0.03*	*4.78±0.44*	*2.39±0.22*
NP35	*316*	*-0.71±0.05*	*5.43±0.27*	*1.09±0.05*
NP36	*360*	*-0.85±0.03*	*7.46±0.39*	*0.74±0.04*

3.2.DSC analysis:

DSC thermograms of drug powder, drug-free and loaded PCL nanoparticles are shown in Fig. 2. DSC experiments were performed to define the physical state of the drug and the polymer

in the formulation. It can be seen that thermograms of drug-free nanoparticles and loaded nanoparticles are identical with an endothermic peak at 59°C which corresponds to the melting point of the polymer. The endothermic peak of the active molecule at 122°C which corresponds to the loss of the crystalline water was absent in both of these thermograms. In such conditions, Yadav and Sawant (2010), who prepared PLGA particles loaded with etoposide, suggested that active molecule is present in an amorphous state inside loaded nanoparticles and that it was homogeneously dispersed in the polymer matrix. In our case, it seems that such conclusion in not probable. ALD is hydrophilic and PCL is hydrophobic. It looks more probable that active is either trapped inside particles or adsorbed to the surface.

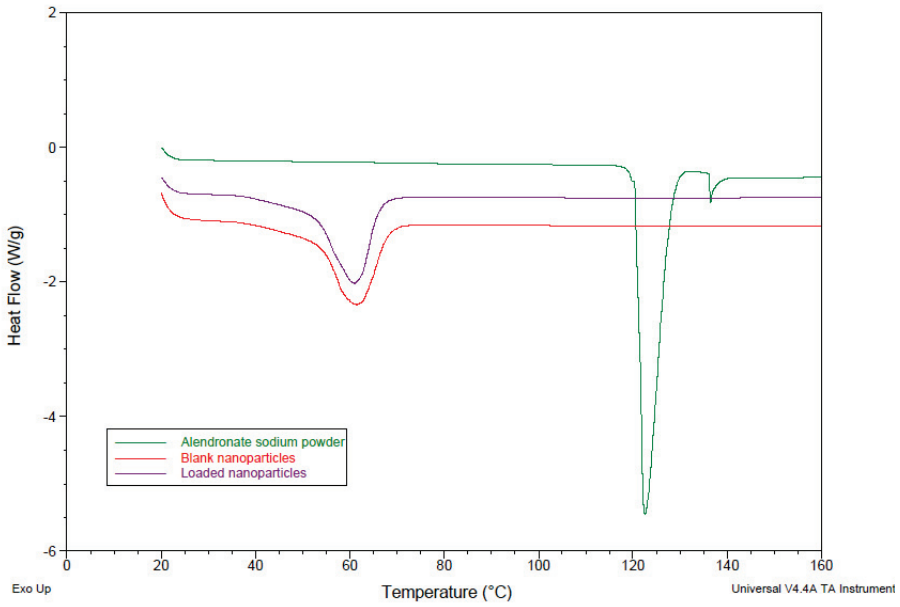


Fig. 2. DSC thermograms of sodium alendronate powder, blank and loaded nanoparticles for formulation DE8

3.3. The influence of organic to water phase volume ratio:

Three different oil to water phase ratios (O/W) were assessed which are 1:2.5, 1:3 and 1:3.5. Influence of O/W ratio is displayed in Fig. 3. An increase of the water phase volume resulted in a significant decrease of particle size ($p<0.05$). For example, particle size decreased from 360 nm for formulation NP4 (O/W=1:2.5) to 238 nm for formulation NP12 (O/W=3.5). This could be explained by the more rapid diffusion of the water-miscible solvent to the aqueous phase which led to quick polymer precipitation and nucleation and thus, the obtaining of smaller nanoparticles. Fonseca et al. (2002) prepared poly(lactide-co-glycolide) (PLGA) nanoparticles and reported that doubling the aqueous phase volume led to a significant

decrease in particle size. Aqueous phase volume augmentation resulted also in a diminution of encapsulation efficiency. In fact, encapsulation efficiency decreased from 18.8% for formulation NP28 (O/W=1:2.5) to 7.46% for formulation NP36 (O/W=1:3.5). This could be explained by a decrease of the concentration of the active molecule in the aqueous phase which rendered it less available for encapsulation.

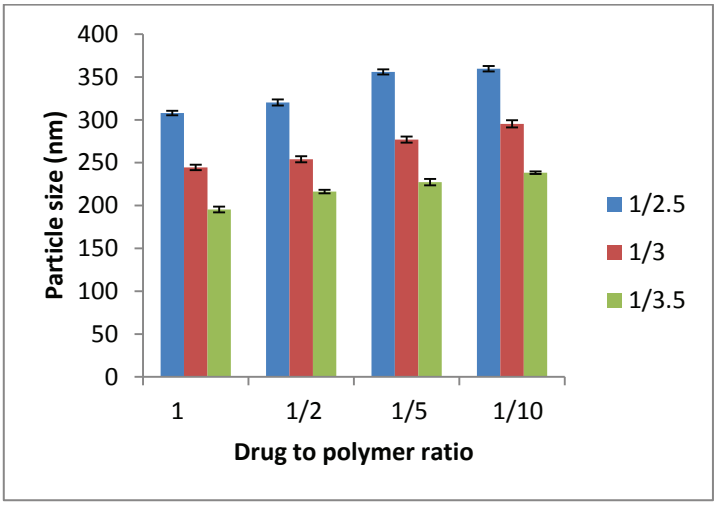


Fig. 3. Influence of oil to water phase ratio in nanoprecipitation for particles prepared with PCL 14000

3.4. The influence of drug to polymer ratio:

Influence of drug to polymer ratio on particle size and encapsulation efficiency is shown by Fig. 4 and Fig. 5. For both techniques, an increase of the polymer amount and thus, the decrease of drug to polymer ratio resulted in a significant increase of particle size and encapsulation efficiency ($p<0.05$). Particle size and encapsulation efficiency increased respectively from 211nm and 15.12% to 272nm and 23.76% when drug to polymer ratio augmented from 1:1 (formulation DE1) to 1:10 (formulation DE4). These findings are consistent with those reported by Chorny et al. (2002) in the case of nanoprecipitation and with those of Van de Ven et al. (2011) in the case of double emulsion. Effect of polymer amount on particle size could be explained by an increase of organic solvent viscosity following the use of higher polymer amount which resulted in the obtaining of bigger droplets. Likely for double emulsion, higher polymer amount could lead to an increased frequency of collisions, resulting in fusion of semiparticles and finally producing bigger particles (Dhanaraju et al. 2004). In nanoprecipitation, enhancement of encapsulation efficiency following drug to polymer ratio increase could be also explained by an increase of

the organic phase viscosity. This could increase the diffusional resistance to drug molecules from organic phase to the aqueous phase and allowed entrapping of higher drug amount in nanoparticles (Budhian et al. 2007). The same phenomenon could restrict migration of drug from inner aqueous phase to the external water phase in the case of double emulsion (Dhanaraju et al. 2004). More efficient entrapment of ALD could be also due to the augmented polymer amount that constructs the matrix of nanoparticles.

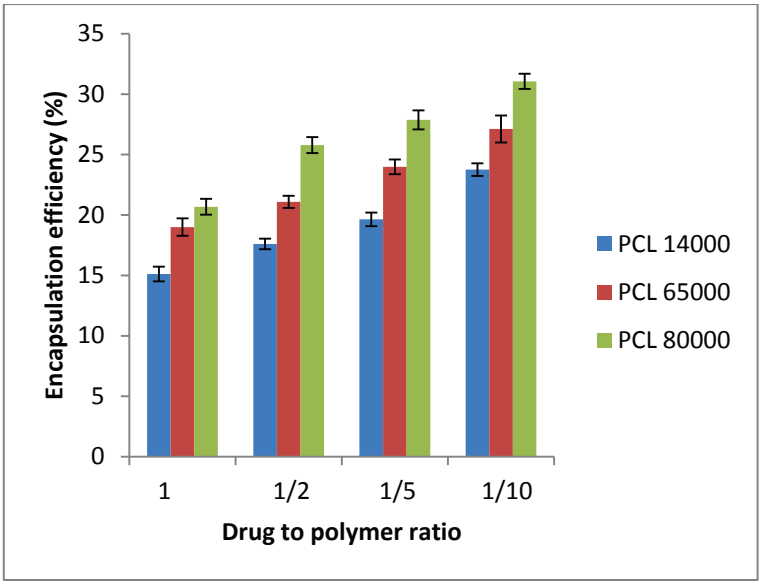


Fig. 4. Influence of drug to polymer ratio on encapsulation efficiency for double emulsion (W/O/W ratio: 1/10/20)

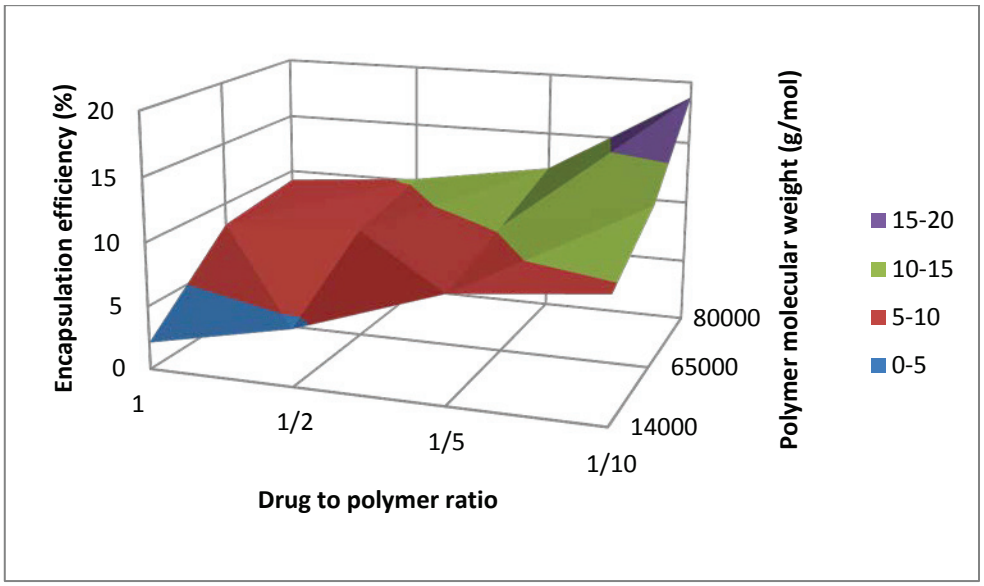


Fig. 5. Variations of encapsulation efficiency in nanoprecipitation technique for water to oil phase ratio of 1/2.5

3.5. The influence of polymer molecular weight:

Fig. 6 and Fig. 7 show influence of polymer molecular weight variations on particle size and encapsulation efficiency. The increase of polymer molecular weight resulted in a significant increase of particle size and encapsulation efficiency for both methods ($p<0.05$). Particle size increased from 272 nm (formulation DE4) to 316 nm (formulation DE8) then to 368 nm (formulation DE12) when polymer molecular weight was increased to 65000 then 80000 g/mol. For nanoprecipitation technique, the same effect on particle size was also reported by Holgado et al. (2012). This effect of polymer molecular weight on particle size and encapsulation efficiency could be explained by an increase of the organic phase viscosity which rendered solvent diffusion more difficult and thus, led to bigger nanoparticles. Such particles could then contain more encapsulated drug. Obtained results could also be explained by more efficient drug encapsulation due to higher polymerization degree of the polymer.

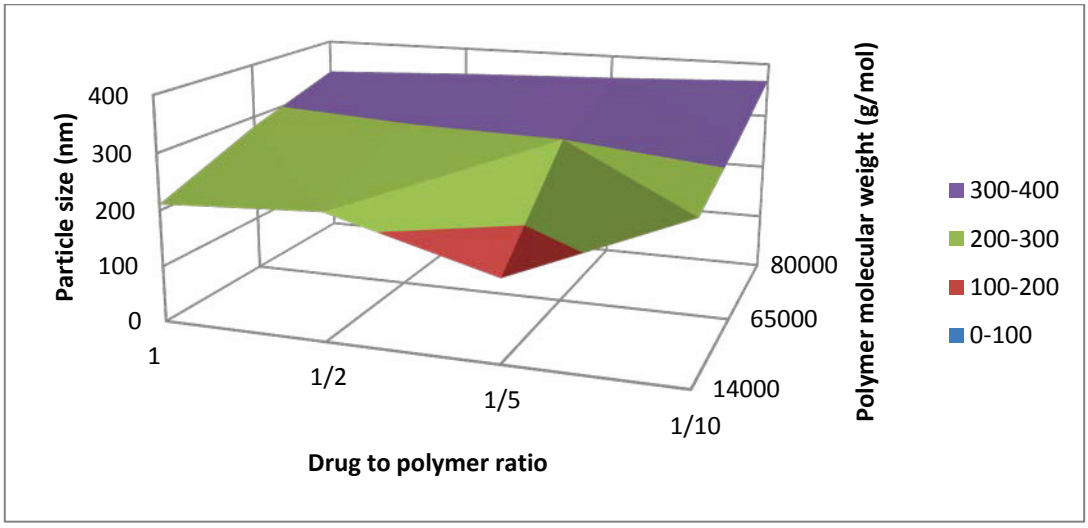


Fig. 6. Particle size variations in double emulsion technique following changes in drug to polymer ratio and polymer molecular weight

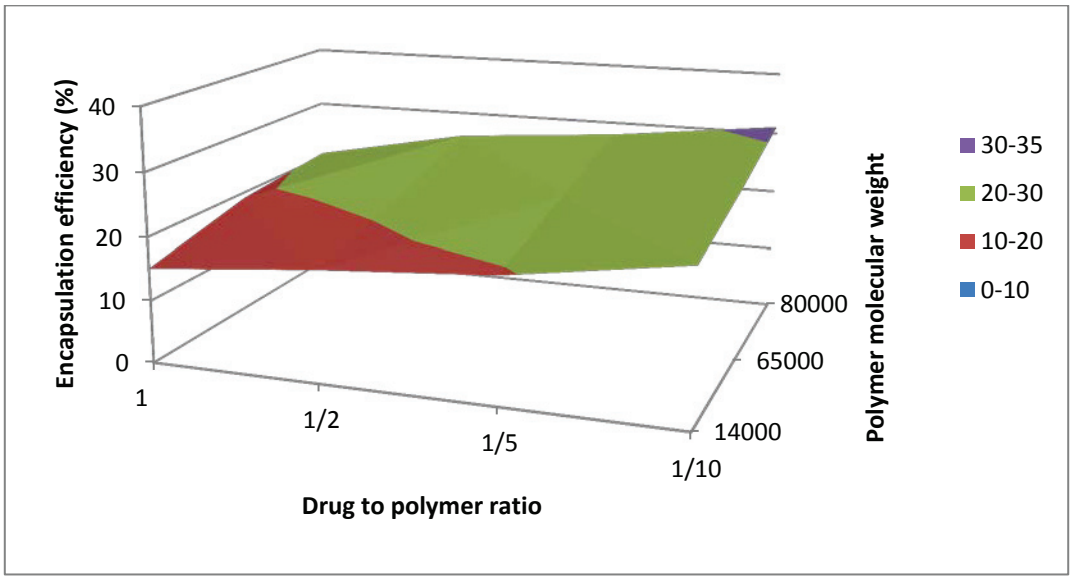


Fig. 7. Encapsulation efficiency variations in double emulsion method following changes in drug to polymer ratio and polymer molecular weight

3.6. The influence of stabilizer amount:

Influence of stabilizer amount on particle size and encapsulation efficiency is presented in Fig. 8. An increase of stabilizer amount led to a non significant decrease of particle size ($p>0.05$). For formulation DE4, particle size decreased from 301 nm to 255 nm when PVA concentration was increased from 0.25% to 1%, respectively. This finding could be explained by an increase of aqueous phase viscosity following PVA concentration increase. Effect of PVA amount on particle size is confirmed by studies carried out by Contado et al. (2013). An increase of encapsulation efficiency was also obtained following an increase of PVA concentration but not for all the formulations. When PVA concentration increased from 0.25% to 1%, encapsulation efficiency increased from 12.18% to 19.03% for formulation NP28. It seems that the presence of more PVA on particles surface hampered active diffusion to the external phase which increased encapsulation efficiency. An increase of PVA amount resulted also in an increase of the zeta potential which could be attributed to an adsorption of PVA on hydrophobic surfaces of PCL nanoparticles which decreased the number of negative charges. This is in agreement with the concept of (Lankveld and Lyklema 1972) which supposes a possible multilayer adsorption of PVA on hydrophobic surfaces.

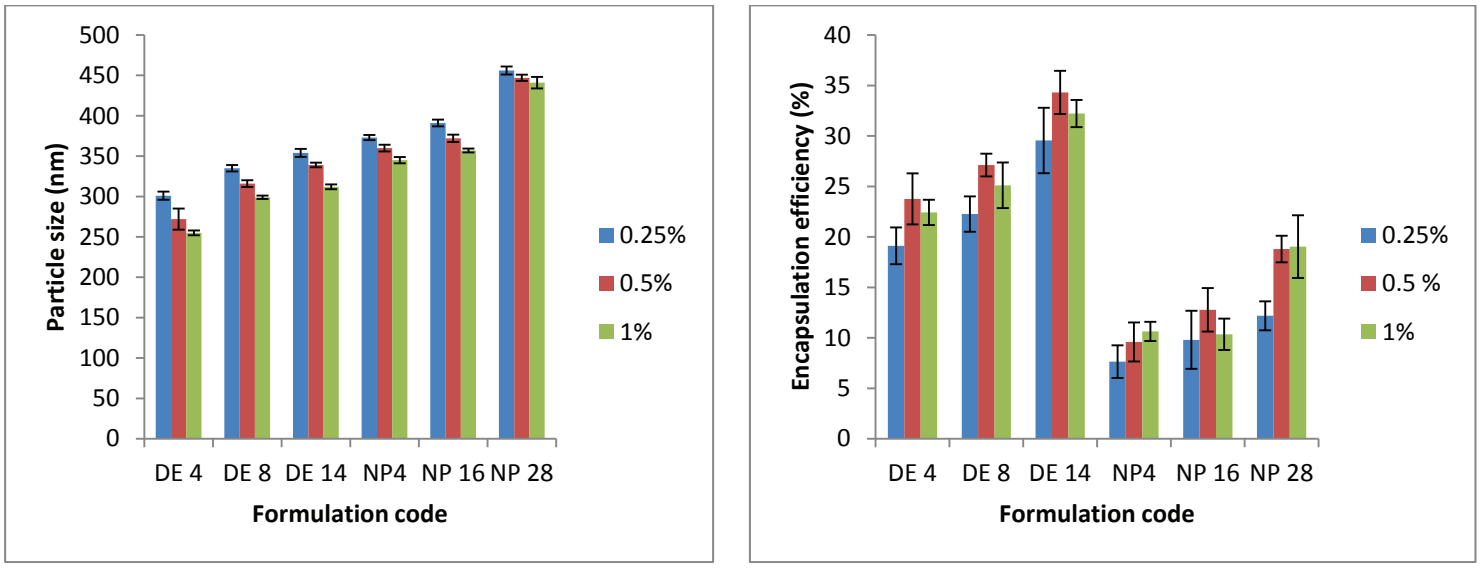


Fig. 8. Influence of stabilizer amount on particle size and encapsulation efficiency

3.7. The influence of outer phase composition:

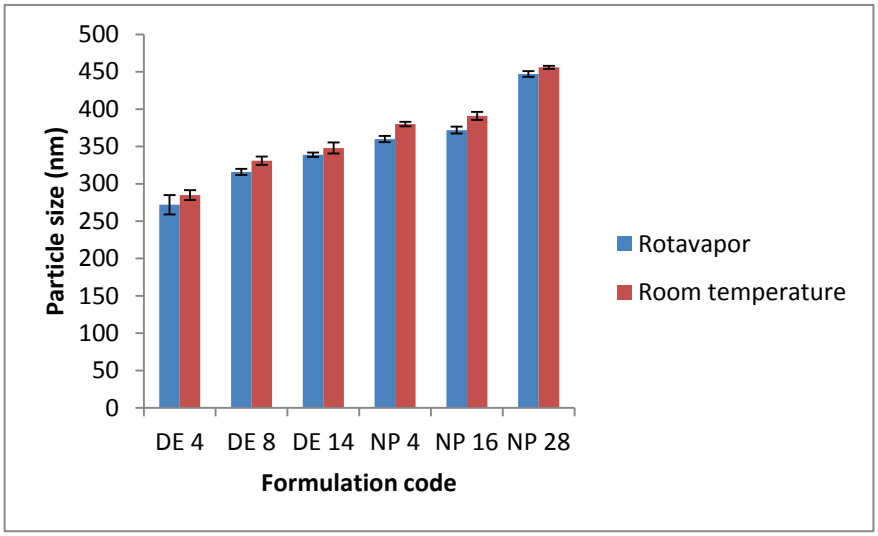
This parameter was assessed for the double emulsion technique by adding different amounts of NaCl in the external aqueous phase. An increase of NaCl amount to 1% resulted in a slight decrease of particle size from 351 nm (DE13) to 339 nm (DE14) and a slight increase of encapsulation efficiency from 32.1% to 34.31%. Decrease of particle size could be attributed to water diffusion from the internal to the external aqueous phase in accordance with the osmotic gradient, leading to shrinkage of the internal aqueous droplets and thus, a decrease in the particle size. In fact, Florence and Whitehill (1982) reported that organic phase of a w/o/w emulsion could act as semipermeable membrane that allows passage of water across the organic phase. Enhancement of encapsulation efficiency following NaCl addition could be explained by the fact that NaCl in the external phase acted as osmogen and provided an effective mechanical barrier to drug diffusion. However, size increased drastically above 1.5% concentration (DE16) to 430 nm while encapsulation efficiency decreased to 25.44%. In addition, particulate carriers' suspension became no longer homogeneous. This could be explained by a disturbing of the ionic forces in the medium by the added electrolyte which led to particles' aggregation and the obtaining of bigger particles.

3.8. The influence of evaporation method and pH:

Rotavaporation (under high temperature and reduced pressure conditions) and evaporation under magnetic stirring at ambient temperature were assessed. It was concluded that rotavapor gave smaller particles' size and better encapsulation efficiency (Fig. 9). However, this

influence was not significant ($p>0.05$). Particle size decreased from 391 nm to 372 nm for formulation NP16 when rotavapor was used. Encapsulation efficiency decreased from 34.31% to 25.41% for the same formulation. Particle size decrease provided by rotavaporation could be explained by rapid nucleation of organic phase droplets while enhancement of encapsulation efficiency could be due to less drug diffusion to the outer aqueous phase.

pH Influence on particles zeta potential was also assessed. Zeta potential remained negative because of the presence of carboxylic groups in polymer structure. pH did not exert significant effect on particles zeta potential ($p>0.05$). An increase of medium pH resulted in a slight decrease of the zeta potential of the particles. For instance, zeta potential decreased from -8.2 at pH2 to -8.93 mV at pH12 in the case of formulation NP4. It decreased also from -10.12 mV to -11.03 mV in the case of formulation DE4. This could be attributed to the increase of negative charges number carried by PCL following alcalinization of the medium.



A

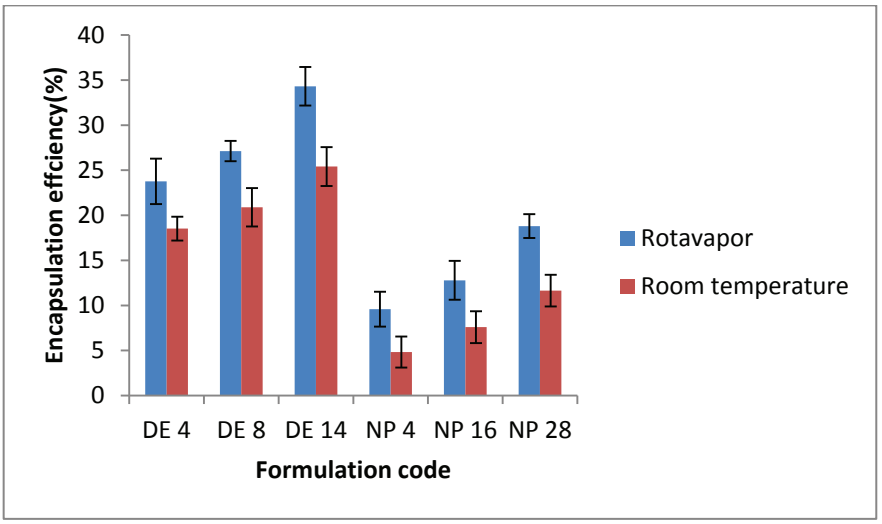


Fig. 9. Influence of evaporation mode on particle size (A) and encapsulation efficiency (B)

3.9.In vitro release studies:

In vitro release of ALD was investigated for 3 selected formulae for each method (DE4, DE8 and DE14 for double emulsion and NP4, NP16 and NP28 for nanoprecipitation). Obtained data were plotted in curves that are shown in Fig. 10. It could be concluded from *in vitro* release profile that the prepared nanoparticles ensured sustained release of the active molecule. This finding was more pronounced for PCL 65000 and PCL 80000. PCL14000 nanoparticles presented faster release and more pronounced burst effect than the other formulations. At 300 minutes more than 60 % of the encapsulated ALD was released by DE 4 while only 35.6% and 31.3% were released by DE8 and DE14, respectively. These findings confirm more sustained release for high molecular weight polymer based particles (Mittal et al. 2007). Moreover, burst release was more pronounced in the case of particles prepared by nanoprecipitation. At the same polymer molecular weight and amount, particles prepared by nanoprecipitation released ALD faster than particles prepared by double emulsion technique. Within the same preparation technique, formulations prepared with PCL 65000 and 80000 showed a very similar release pattern, although they differ in particle size. Consequently, main difference with PCL 14000 would mainly stem from the presence of a more compact and less permeable polymer matrix which made their release profiles almost superimposable. Furthermore, all formulations provided the same kinetic profile drug release which was characterized by a first rapid phase followed by a second slower phase. This initial burst phenomenon might be due to the rapid release of ALD deposited on the surface and in the water channels of nanoparticles (Perez et al. 2001). *In vitro* active release from nanoparticles

holding the same drug and constructed by the same polymer depends on many parameters: polymer concentration, oil nature, particle size, *in vitro* release test onditions (medium pH and temperature) and preparation conditions. In the case of our study, although nanoparticles obtained by double emulsion were smaller, they didn't release ALD in a faster time. These results are not consistent with those reported by Zili et al. (2005) who attributed faster release of smaller particles to the more available surface area. In addition, ALD was not completely delivered even in the case of nanoparticles prepared by the nanoprecipitation which gave faster release. This incomplete release of the active could be explained by the retention capacity of the active substance by the polymer (Cauchetier et al. 2003).

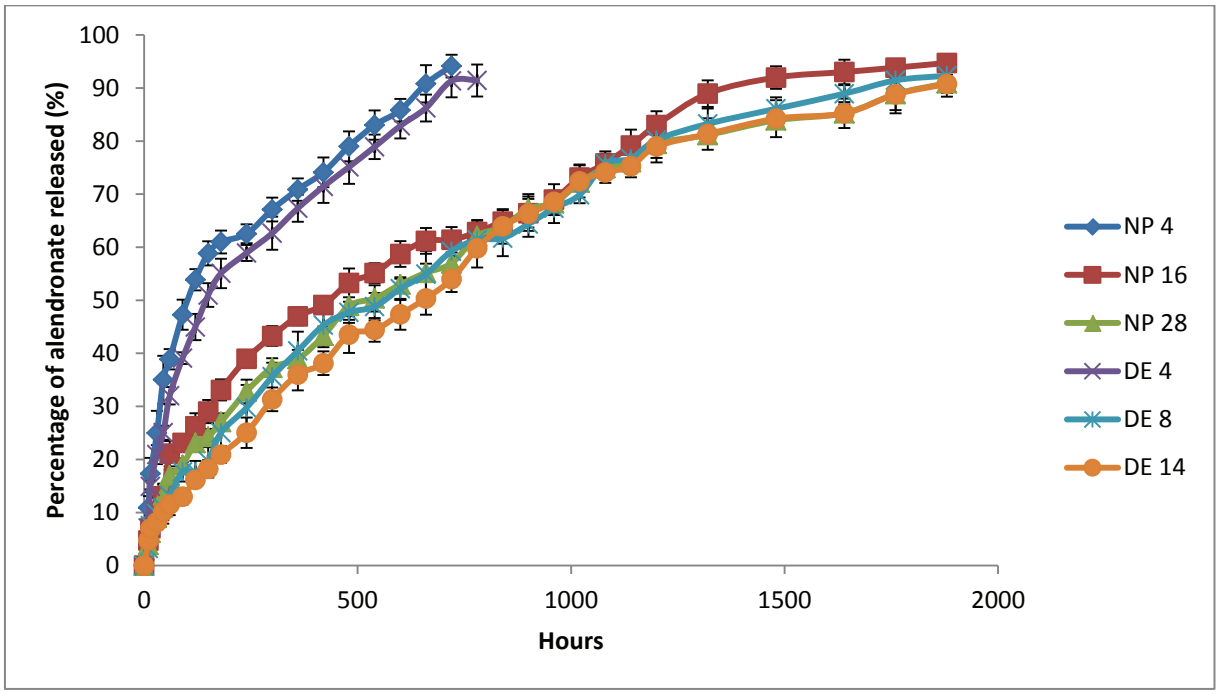


Fig. 10. In vitro release of alendronate from nanoparticles

To further understand the phenomena involved in drug release process, obtained data were analyzed by two *in vitro* release models: the Higuchi model and the Krosmeyer-Pepas model. The Higuchi model is a mathematical model that was proposed by Higuchi in 1961 to describe drug release from a matrix system. This model is expressed by equation (a).

$$Qt = A\sqrt{D(2C0 - Cs)Cs\,t} \qquad \text{(a)}$$

Where Q_t is the amount of the drug released in time t from unit area of surface A. C0 is the initial drug concentration. Cs is the drug solubility in the matrix media and D is the diffusivity of the drug molecule in the matrix. Higuchi model describes drug release as a diffusion process based on Fick's law, which is square root of time dependent. Therefore, when release kinetics fit this model, active release from particles would be mainly controlled by diffusion through polymer matrix.

To gain further insight into the mechanisms that govern drug release, obtained data were also analyzed by the Korsmeyer-Peppas model, particles being considered as spheres based on morphological data (Sanna et al. 2011). The Korsmeyer-Peppas model is a semiempirical model generally used to analyze release data of different pharmaceutical dosage forms and it is expressed by equation (b).

$$\frac{Mt}{M\infty} = kt^n \qquad \text{(b)}$$

where *Mt* and *M*∞ are the absolute cumulative amount of drug released at time t and at infinite time, respectively. *k* is the constant incorporating structural and geometric characteristics of the release device and *n* is the release exponent, indicative of the mechanism of drug release. To determine *n*, only the curve release fraction of M*t*/M∞ ≤ 0.6 was used. In Korsmeyer-Peppas model, *n* values of 0.43 indicate that the drug release is controlled by Fickian diffusion. Conversely, *n* values between 0.43 and 0.85 imply a non-Fickian diffusion process which is also known as anomalous transport. The latter could be described as a combination of drug diffusion and polymer chain relaxation as long as the solvent diffuse into the polymeric matrix. However, if n ≥ 0.85, this indicates that drug release is only governed by polymer relaxation (Puga et al. 2012).

In all cases, release kinetics fitted the Higuchi model with R2 values higher than 0.96 which suggests that active release was mainly controlled by diffusion (See Table 5). Moreover, all investigated formulations fitted Korsmeyer-Peppas model with R2 values > 0.97 and obtained n values were in the 0.5684-0.6016 range. Consequently, drug release was accomplished by combination of two phenomena: drug diffusion and polymer chain relaxation.

Table 5. Curve fitting analysis of ALD-loaded particles

Formulation	Higuchi model	Korsmeyer-Peppas model	
	R^2	R^2	*n*
DE 4	0.9833	0.9719	0.5795
DE 8	0.994	0.9858	0.5774
DE 14	0.9874	0.9825	0.5684
NP 4	0.961	0.9809	0.6016
NP 16	0.9925	0.9837	0.5918
NP 28	0.9955	0.9888	0.5899

4. Conclusion:

Osteoporosis is a chronic metabolic disease that affects bone and leads to high rates of morbidity and mortality. We prepared polycaprolactone based nanoparticles loaded with alendronate which is a bisphosphonate used as first-line regimen for the management of osteoporosis. Nanoparticles were prepared by two techniques: double emulsion and nanoprecipitation. Preparation method and operational conditions exerted great effect on the characteristics of the obtained nanoparticles. Higher encapsulation efficiency and drug loading values were obtained with the double emulsion method. Furthermore, *in vitro* release studies of the active pharmaceutical ingredient showed almost the same kinetic profiles but, in spite of their smaller mean diameter, more pronounced release of the encapsulated drug was recorded in formulations prepared by double emulsion evaporation method. It was also shown that polymer molecular weight strongly affected the release behavior of alendronate. Analysis of *in vitro* release data showed also that alendronate release mechanism from polycaprolactone nanoparticles relied on a combination of drug diffusion and polymer relaxation phenomena. Further studies are required to enhance encapsulation efficiency values and to obtain a more rapid release of alendronate. *In vivo* studies should also be performed to confirm the efficacy of prepared nanoparticles.

Acknowledgments:

K. Miladi would like to acknowledge the support provided by Tunisian Ministry Of Higher Education And Scientific Research by awarding a grant.

References:

Bilati U, Allémann E, Doelker E (2005) Development of a nanoprecipitation method intended for the entrapment of hydrophilic drugs into nanoparticles. European Journal of Pharmaceutical Sciences 24:67–75. doi: 10.1016/j.ejps.2004.09.011

Budhian A, Siegel SJ, Winey KI (2007) Haloperidol-loaded PLGA nanoparticles: systematic study of particle size and drug content. Int J Pharm 336:367–375. doi: 10.1016/j.ijpharm.2006.11.061

Campos EVR, de Melo NFS, de Paula E, et al. (2013) Screening of Conditions for the Preparation of Poly(-Caprolactone) Nanocapsules Containing the Local Anesthetic Articaine. Journal of Colloid Science and Biotechnology 2:106–111. doi: 10.1166/jcsb.2013.1040

Cauchetier E, Deniau M, Fessi H, et al. (2003) Atovaquone-loaded nanocapsules: influence of the nature of the polymer on their in vitro characteristics. Int J Pharm 250:273–281.

Chorny M, Fishbein I, Danenberg HD, Golomb G (2002) Lipophilic drug loaded nanospheres prepared by nanoprecipitation: effect of formulation variables on size, drug recovery and release kinetics. Journal of Controlled Release 83:389–400. doi: 10.1016/S0168-3659(02)00211-0

Cohen-Sela E, Chorny M, Koroukhov N, et al. (2009) A new double emulsion solvent diffusion technique for encapsulating hydrophilic molecules in PLGA nanoparticles. J Control Release 133:90–95. doi: 10.1016/j.jconrel.2008.09.073

Cohen-Sela E, Rosenzweig O, Gao J, et al. (2006) Alendronate-loaded nanoparticles deplete monocytes and attenuate restenosis. J Control Release 113:23–30. doi: 10.1016/j.jconrel.2006.03.010

Contado C, Vighi E, Dalpiaz A, Leo E (2013) Influence of secondary preparative parameters and aging effects on PLGA particle size distribution: a sedimentation field flow fractionation investigation. Anal Bioanal Chem 405:703–711. doi: 10.1007/s00216-012-6113-5

Cremers, S.C., Pillai, G., Papapoulos, S.E., 2005. Pharmacokinetics/pharmacodynamics of bisphosphonates: use for optimisation of intermittent therapy for osteoporosis. Clin Pharmacokinet 44, 551–570.

Dash TK, Konkimalla VB (2012) Poly-ϵ-caprolactone based formulations for drug delivery and tissue engineering: A review. Journal of Controlled Release 158:15–33. doi: 10.1016/j.jconrel.2011.09.064

De Araújo TM, Teixeira Z, Barbosa-Sampaio HC, et al. (2013) Insulin-loaded poly(epsilon-caprolactone) nanoparticles: efficient, sustained and safe insulin delivery system. J Biomed Nanotechnol 9:1098–1106.

De Melo NFS, Campos EVR, de Paula E, et al. (2013) Factorial Design and Characterization Studies for Articaine Hydrochloride Loaded Alginate/Chitosan Nanoparticles. Journal of Colloid Science and Biotechnology 2:146–152. doi: 10.1166/jcsb.2013.1048

Dhanaraju MD, Jayakumar R, Vamsadhara C (2004) Influence of manufacturing parameters on development of contraceptive steroid loaded injectable microspheres. Chem Pharm Bull 52:976–979.

El-Aasser MS, Ugelstad J, Vanderhoff JW (1979) Polymer emulsification process.

Ezra A, Golomb G (2000) Administration routes and delivery systems of bisphosphonates for the treatment of bone resorption. Adv Drug Deliv Rev 42:175–195.

Fasinu P, Pillay V, Ndesendo VMK, et al. (2011) Diverse approaches for the enhancement of oral drug bioavailability. Biopharm Drug Dispos 32:185–209. doi: 10.1002/bdd.750

Fessi H, Puisieux F, Devissaguet JP, et al. (1989) Nanocapsule formation by interfacial polymer deposition following solvent displacement. International Journal of Pharmaceutics 55:R1–R4. doi: 10.1016/0378-5173(89)90281-0

Florence AT, Whitehill D (1982) The formulation and stability of multiple emulsions. International Journal of Pharmaceutics 11:277–308. doi: 10.1016/0378-5173(82)90080-1

Fonseca C, Simões S, Gaspar R (2002) Paclitaxel-loaded PLGA nanoparticles: preparation, physicochemical characterization and in vitro anti-tumoral activity. J Control Release 83:273–286.

Grando CRC, Guimarães CA, Mercuri LP, et al. (2013) Preparation and Characterization of Solid Lipid Nanoparticles Loaded with Racemic Mitotane. Journal of Colloid Science and Biotechnology 2:140–145. doi: 10.1166/jcsb.2013.1044

Han S, Li M, Liu X, et al. (2013) Construction of amphiphilic copolymer nanoparticles based on gelatin as drug carriers for doxorubicin delivery. Colloids Surf B Biointerfaces 102:833–841. doi: 10.1016/j.colsurfb.2012.09.010

Holgado MA, Martin-banderas, Alvarez-fuentes, et al. (2012) Cannabinoid derivate-loaded PLGA nanocarriers for oral administration: formulation, characterization, and cytotoxicity studies. International Journal of Nanomedicine 5793. doi: 10.2147/IJN.S34633

Lankveld JM., Lyklema J (1972) Adsorption of polyvinyl alcohol on the paraffin—water interface. II. Spread and adsorbed monolayers. Journal of Colloid and Interface Science 41:466–474. doi: 10.1016/0021-9797(72)90370-0

Lindfors L, Skantze P, Skantze U, et al. (2006) Amorphous drug nanosuspensions. 1. Inhibition of Ostwald ripening. Langmuir 22:906–910. doi: 10.1021/la0523661

Lira AAM, Cordo PLA, Nogueira ECF, et al. (2013) Optimization of Topical All-Trans Retinoic Acid Penetration Using Poly-DL-Lactide and Poly-DL-Lactide-Co-Glycolide Microparticles. Journal of Colloid Science and Biotechnology 2:123–129. doi: 10.1166/jcsb.2013.1041

Mazzaferro S, Bouchemal K, Maksimenko A, et al. (2012) Reduced Intestinal Toxicity of Docetaxel Loaded Into Mucoadhesive Nanoparticles, in Mouse Xenograft Model.

Journal of Colloid Science and Biotechnology 1:210–217. doi: 10.1166/jcsb.2012.1022

Mishra PR, Al Shaal L, Müller RH, Keck CM (2009) Production and characterization of Hesperetin nanosuspensions for dermal delivery. Int J Pharm 371:182–189. doi: 10.1016/j.ijpharm.2008.12.030

Mittal G, Sahana DK, Bhardwaj V, Ravi Kumar MNV (2007) Estradiol loaded PLGA nanoparticles for oral administration: Effect of polymer molecular weight and copolymer composition on release behavior in vitro and in vivo. Journal of Controlled Release 119:77–85. doi: 10.1016/j.jconrel.2007.01.016

Ostović D, Stelmach C, Hulshizer B (1993) Formation of a Chromophoric Complex Between Alendronate and Copper(II) Ions. Pharm Res 10:470–472. doi: 10.1023/A:1018969112754

Perez C, Sanchez A, Putnam D, et al. (2001) Poly(lactic acid)-poly(ethylene glycol) nanoparticles as new carriers for the delivery of plasmid DNA. Journal of Controlled Release 75:211–224. doi: 10.1016/S0168-3659(01)00397-2

Puga AM, Rey-Rico A, Magariños B, et al. (2012) Hot melt poly-ε-caprolactone/poloxamine implantable matrices for sustained delivery of ciprofloxacin. Acta Biomaterialia 8:1507–1518. doi: 10.1016/j.actbio.2011.12.020

Riddick TM, inc Z-M (1968) Control of colloid stability through zeta potential: with a closing chapter on its relationship to cardiovascular disease. Published for Zeta-Meter, inc., by Livingston Pub. Co.

Rosset V, Ahmed N, Zaanoun I, et al. (2012) Elaboration of Argan Oil Nanocapsules Containing Naproxen for Cosmetic and Transdermal Local Application. Journal of Colloid Science and Biotechnology 1:218–224. doi: 10.1166/jcsb.2012.1023

Sanna V, Roggio AM, Posadino AM, et al. (2011) Novel docetaxel-loaded nanoparticles based on poly(lactide-co-caprolactone) and poly(lactide-co-glycolide-co-caprolactone) for prostate cancer treatment: formulation, characterization, and cytotoxicity studies. Nanoscale Res Lett 6:260. doi: 10.1186/1556-276X-6-260

Seremeta KP, Chiappetta DA, Sosnik A (2013) Poly(ε-caprolactone), Eudragit® RS 100 and poly(ε-caprolactone)/Eudragit® RS 100 blend submicron particles for the sustained release of the antiretroviral efavirenz. Colloids Surf B Biointerfaces 102:441–449. doi: 10.1016/j.colsurfb.2012.06.038

Sinha VR, Bansal K, Kaushik R, et al. (2004) Poly-epsilon-caprolactone microspheres and nanospheres: an overview. Int J Pharm 278:1–23. doi: 10.1016/j.ijpharm.2004.01.044

Sweetman SC (2009) Martindale: The Complete Drug Reference. Pharmaceutical Press

Van de Ven H, Vermeersch M, Matheeussen A, et al. (2011) PLGA nanoparticles loaded with the antileishmanial saponin β-aescin: factor influence study and in vitro efficacy evaluation. Int J Pharm 420:122–132. doi: 10.1016/j.ijpharm.2011.08.016

Wang F, Li J, Wang C (2012) Hydrophilic and Fluorescent Colloidal Nanorods of MWNTs as Effective Targeted Drug Carrier. Journal of Colloid Science and Biotechnology 1:192–200. doi: 10.1166/jcsb.2012.1024

Yadav KS, Sawant KK (2010) Formulation optimization of etoposide loaded PLGA nanoparticles by double factorial design and their evaluation. Curr Drug Deliv 7:51–64.

Zili Z, Sfar S, Fessi H (2005) Preparation and characterization of poly-epsilon-caprolactone nanoparticles containing griseofulvin. Int J Pharm 294:261–267. doi: 10.1016/j.ijpharm.2005.01.020

Optimisation de l'encapsulation de l'alendronate dans des nanoparticules de chitosane

Publiée *sous forme d'un article scientifique dans*

« Journal of Drug Delivery Science and Technology »

Journal of Drug Delivery Science and Technology *2015*

Cet article comprend une étude d'encapsulation d'alendronate de sodium dans des nanoparticules à base de chitosane. Cette étude est divisée en trois parties. Une première partie dédiée à l'étude systématique et une deuxième partie à la caractérisation des nanoparticules préparées. Dans la troisième partie, on s'intéresse à la libération *in vitro* de la substance active à partir des nanoparticules de chitosane.

Les nanoparticules de poly-ε-caprolactone qui ont été précédemment préparées par émulsion double et nanoprécipitation ont présenté des propriétés intéressantes. Cependant, le pourcentage d'encapsulation obtenu reste faible. En plus, les études *in vitro* ont montré une libération très prolongée de l'alendronate. Ceci peut être intéressant pour minimiser le nombre de prises journalières. Mais au-delà d'une certaine limite, une telle libération n'aura pas d'intérêt en clinique. De ce fait, cette deuxième partie expérimentale vise à pallier les inconvénients obtenus avec les nanoparticules de poly-ε-caprolactone préparées dans la première partie. Cette étude repose sur l'encapsulation de l'alendronate de sodium dans un polymère hydrophile. Il s'agit du chitosane qui est un polymère naturel présentant des propriétés intéressantes en encapsulation. Il a été utilisé comme matrice ou bien en tant que « coating ». En effet, les charges positives que porte ce polymère permettent une forte interaction électrostatique avec la muqueuse gastro-intestinale (chargée négativement grâce à la présence de la mucine) ce qui augmenterait l'absorption des substances actives et donc leurs biodisponibilités. La technique utilisée pour la préparation des nanoparticules est la gélification ionique. Cette méthode possède l'avantage d'être simple et de ne pas nécessiter l'utilisation de solvants organiques. La technique de gélification ionique est basée sur le passage d'un polymère dissous à la forme gel. Dans notre cas, ce changement d'état est du à une interaction électrostatique entre le chitosane (chargé positivement) et le tripolyphosphate de sodium (chargé négativement). Expérimentalement, l'alendronate est dissout dans la solution de chitosane. Par la suite, la solution du tripolyphosphate de sodium est ajoutée goutte à goutte à la solution du chitosane sous agitation magnétique. La taille des particules obtenues a varié entre 91 et 175 nm. Les images de la microscopie électronique à transmission ont montré des nanoparticules de forme sphérique régulière L'étude systématique a montré qu'à des ratios égaux, une augmentation de la concentration du chitosane et du tripolyphosphate de sodium entraine une augmentation de la taille des particules. L'effet du

ratio chitosane/tripolyphosphate était variable. Lorsque la concentration du chitosane et tripolyphosphate était de 1 mg/ml, l'effet du changement du ratio était variable. Par contre, à une concentration de chitosane et de tripolyphosphate égale à 2 mg/ml, une augmentation constante de la taille a été observée. Le potentiel Zêta des particules a été positif et les valeurs étaient entre 21 et 27 mV. Ces valeurs positives s'expliquent par la présence de groupements ammonium quaternaires à la surface des particules qui sont apportés par le chitosane. A des ratios chitosane/tripolyphosphate égaux, une tendance générale d'augmentation du potentiel Zêta a été notée. Par ailleurs, l'augmentation du ratio chitosane/tripolyphosphate a entrainé le même effet. Ceci peut être expliqué par une augmentation des charges positives suite à l'addition du chitosane. Une augmentation du pourcentage d'encapsulation a été également observée suite à une augmentation de la concentration du chitosane et du tripolyphosphate en conservant les mêmes ratios. L'augmentation de la concentration du chitosane (chargé positivement) pourrait être à l'origine d'une interaction plus forte avec l'alendronate (chargé négativement). Ceci s'est traduit par une tendance générale d'augmentation du pourcentage d'encapsulation. La valeur maximale du pourcentage d'encapsulation obtenue a été de 70%. De ce fait, une amélioration nette du pourcentage d'encapsulation a été obtenue par rapport à la première étude où un taux maximal de 34% a été observé. Cette amélioration peut être expliquée par une plus forte interaction substance active-polymère. En effet, l'alendronate et le chitosane sont tous les deux de nature hydrophile. En plus, ils possèdent des charges opposées. Les particules préparées ont été aussi analysées par spectroscopie infrarouge à transformée de Fourier. Ces analyses ont confirmé la présence d'alendronate à l'intérieur des particules ainsi que la formation de complexes entre le chitosane et le tripolyphosphate. L'étude de la libération *in vitro* a permis de déceler une différence de diffusion qui dépend du pH du milieu. En effet, la libération de l'alendronate a été plus rapide en milieu HCl à 0,1N (pH=1,2) qu'en milieu tampon phosphate pH6,8. Ceci s'explique par la meilleure solubilité du chitosane à pH acide. En milieu HCl, l'équilibre a été atteint après 60 min alors que 240 min ont été nécessaires pour atteindre l'équilibre dans la solution du tampon phosphate pH6,8. Ceci a permis de mettre en évidence le rôle de pH du milieu dans la diffusion de la substance active à partir des particules. Le profil général de libération a été, cependant, similaire avec une phase de libération rapide qui est suivie par une phase de libération plus lente. La phase de libération rapide ou de « burst release » serait due à l'alendronate qui est adsorbé à la surface des nanoparticules. Lorsque le chitosane était insoluble à pH neutre, la deuxième phase a été plus prolongée que celle qui correspond au pH acide. La réalisation d'études *in*

vivo permettrait d'estimer l'efficacité thérapeutique des nanoparticules préparées. Un bénéfice en matière de tolérance pourrait aussi être cherché.

Enhancement of alendronate encapsulation in chitosan nanoparticles

K. Miladi[a,b], S. Sfar[b], H. Fessi[a]*, A. Elaissari[a]

[a] University of Lyon, Lyon, France; Lyon 1 University, Villeurbanne, CNRS, UMR 5007, Laboratoire d'Automatique et de Génie des Procédés, LAGEP-CPE-308G, 43 bd. du 11 Nov.1918, F-69622, Villeurbanne, France.

[b] University of Monastir, Laboratoire de pharmacie galénique, Rue Avicenne, 5000 Monastir, Tunisia

* Corresponding author: Phone: +33-472431841, Fax: +33-472431682

E-mail: fessi@lagep.univ-lyon1.fr

Abstract:

The encapsulation of active molecules in chitosan has gained an increasing interest during the last decades. Chitosan is regarded as biodegradable and safe material, which is suitable for drug delivery. However, owing to the pronounced variability between chitosan types and sources, data are still lacking about optimal conditions for the preparation of chitosan nanoparticles. In this study, we assess influence of some experimental parameters on the encapsulation of alendronate sodium. The latter is an active molecule, which is used as first line regimen for the treatment of osteoporosis. Chitosan nanoparticles were prepared by the ionic gelation technique and then characterized. Obtained particle size was in the range of 91-175 nm and Zeta potential ranged between +21 and +27 mV depending on the formulation. TEM images showed spherical nanoparticles with a compact structure. The highest encapsulation efficiency was 70%. *In vitro* release profiles depended on the dissolution medium. Nanoparticles released alendronate faster in 0.1N HCl than in phosphate buffer pH6.8. The proposed nanoparticles offer an interesting alternative for alendronate delivery via the oral route.

Keywords:

Alendronate sodium, chitosan, nanoparticles, ionic gelation, osteoporosis, *in vitro*.

1. Introduction:

Alendronate sodium (ALD) is an active molecule which is indicated as first line regimen for the treatment of osteoporosis. Efficacy of this active molecule is well proven but therapy presents some limitations such as, low oral bioavailability and gastrointestinal side effects [1]. During the last decades, several studies have focused on encapsulation of active molecules as a tool to enhance treatment efficiency and to minimize side effects [2][3][4]. Several techniques were also used such as nanoprecipitation [5], emulsion solvent evaporation [6], emulsion solvent diffusion [7] and ionic gelation [8]. Recently, preparation of polycaprolactone nanoparticles containing ALD via double emulsion and nanoprecipitation techniques was reported by Miladi et al. [9]. Obtained results were encouraging but major disadvantage of the used techniques was the necessity to add organic solvents like acetone (for nanoprecipitation) and dichloromethane (for double emulsion). In fact dichloromethane is classified as class 2 residual solvent according to International Conference on Harmonization (ICH). Such solvents should be limited in pharmaceutical products and the maximum allowed concentration is 600ppm. Acetone belongs, however, to class 3 residual solvents. Use of such solvents should be limited by Good Manufacturing Practices (GMP) or other Quality-based requirements [10]. For this reason, we decided to use a safer technique. For instance, ionic gelation is a simple and safe method that has been widely used to encapsulate hydrophilic molecules. Used polymers consist, generally, of natural hydrophilic polymers such as chitosan. Chitosan is a cationic and biodegradable polysaccharide consisting of repeating D-glucosamine and Nacetyl- D-glucosamine units, linked via (1-4) glycosidic bonds. It is non toxic and could be digested in the physiological environment, either by lysozymes or by chitinases, which are present in the human intestine and in the blood. These properties led to increased interest for this polymer in pharmaceutical research and industry as a carrier for drug delivery [11]. The technique is based on the transition of the polymer from liquid state to a gel. Chitosan particles could be prepared by several techniques [12]. This is due to the spontaneous formation of complexes between the positively charged chitosan and polyanions (tripolyphosphate or gelatin) or on the gelation of a chitosan solution dispersed in an oil emulsion [13]. Furthermore, the use of hydrophilic polymers like chitosan could be interesting for the encapsulation of hydrophilic molecules such as, alendronate sodium. Chitosan is also well known for its mucoadhesive properties [14][15][16]. When administered via the oral route, chitosan could easily interact with mucin via electrostatic forces thanks to its positive charges. Such an effect could be interesting for molecules that present weak absorption and low diffusion potential through biological membranes [17].

2. Materials and methods:

2.1.Materials:

Alendronate ((4-amino-1-hydroxybutylidene) diphosphonate trihydrate) was purchased from Polpharma, Poland. Low molecular weight chitosan (20-300cP), sodium tripolyphosphate (TPP), sodium hydroxide, acetic acid glacial, fluorenyl-methyloxycarbonyl chloride (FMOC), acetonitrile and dichloromethane were purchased from Sigma-Aldrich. Disodium hydrogen phosphate and trisodium citrate were obtained from VWR. Disodium tetraborate decahydrate was obtained from Merck Millipore. All other reagents were of analytical grade and were used without further purification.

2.2.Methods:

2.2.1. Nanoparticles preparation:

Nanoparticles were prepared by ionic gelation technique. TPP was dissolved in water milliQ. Two TPP solutions at different concentrations were prepared: 1mg/ml and 2mg/ml. Chitosan was dissolved in water milliQ containing acetic acid. Two chitosan solutions were prepared: one at a concentration of 1mg/ml that contains 1% v/v acetic acid and the other at 2mg/ml and contains 2% v/v acetic acid. Both solutions have been kept under magnetic agitation overnight to allow complete dissolution of chitosan. Then, pH of both chitosan solutions was fixed at pH4.7 by the addition of 0.1M NaOH solution. Nanoparticles were prepared by first dissolving 20mg of ALD in 10ml of chitosan solution. Then, different TPP amounts were added dropwise to 10 ml of the acidic chitosan solution under magnetic stirring at 250 rpm. Stirring was kept for 15 minutes and preparation was carried at room temperature. Operating conditions that were changed were: TPP concentration, chitosan concentration and chitosan to TPP ratio. Nanoparticles suspension was subsequently subjected to ultracentrifugation at 30000 rpm during 30 minutes by ultracentrifuge Optima (Beckman Coulter, France) to separate the obtained nanocarriers. As **Table 1** shows, 8 different formulations were prepared.

Table 1. *Formulations prepared by ionic gelation*

Chitosan concentration (mg/ml)	TPP concentration (mg/ml)	Formulation code	Ratio chitosan to TPP
1	1	**CH1**	2.50
		CH2	3.33
		CH3	4.00
		CH4	5.00
2	2	**CH5**	2.50
		CH6	3.33
		CH7	4.00
		CH8	5.00

2.2.2. Nanoparticles characterization:

2.2.2.1.Particle size and zeta potential:

Z-average diameter of the prepared nanoparticles and their Zeta potential were determined by Malvern particle size analyzer (Model-Nano ZS, Malvern Instruments limited, UK). The nanoparticles were dispersed in a 1 mM NaCl before each measurement. All measurements were carried out in triplicate at 25°C.

2.2.2.2.TEM images:

Nanoparticles morphology was examined by transmission electron microscopy (TEM) apparatus Philips CM-120 at an accelerating voltage of 100 kV. Nanoparticles suspension was properly diluted. A drop was withdrawn with a micropipette then placed on a carbon-coated copper grid. The excess of the suspension was removed by blotting the grid with a filter paper. Then the deposit was left to dry before analysis.

2.2.2.3.*FTIR analysis:*

FTIR analysis was performed on chitosan powder, alendronate powder, alendronate containing nanoparticles and drug-free nanoparticles. Analysis was carried out by FTIR analyser IRPrestige 21, Schimadzu, Japan.

2.2.2.4.Encapsulation efficiency:

The amount of ALD loaded in nanoparticles was determined by High Performance Liquid chromatography (Agilent 1200 series). The obtained particles were subjected to ultracentrifugation at a speed of 30000 rpm during 30 minutes. ALD amount was then determined in the supernatant using the indirect method. The used column was a Zorbax

Eclipse XDB-C18 column (4,6x150mm,5µ particle size). Mobile phase was a mixture of acetonitrile, methanol and 0.05M disodium hydrogeno-phosphate/0.05M citrate trisodium (20:5:75, pH8). Mobile phase flow rate was set at 1ml/mn and UV detection wavelength was fixed at 266 nm. A derivatization reaction was performed before injection. 5 ml of each sample was collected in a 50ml polypropylene centrifuge tube. 5ml of 0.1M aqueous disodium tetraborate decahydrate solution was added. Then 5 ml of 1mg/ml FMOC solution in acetonitrile was added. Tube was vortexed for 30s and reaction was allowed for 30min. Subsequently, 25ml of dicholoromethane was added and tube was shaken for 30-60s then allowed to stand for 5 min. After this, tube was centrifuged at 2000 rpm for 10 min to remove excess reagent. Finally, a portion of the top layer was removed by a syringe then filtered and transferred to a HPLC vial. Encapsulated drug amount was obtained by subtraction of the amount of active molecule in the supernatant from the initial amount of ALD. Analytical method was validated by the investigation of linearity, precision, specificity and accuracy. Encapsulation efficiency was expressed as encapsulated drug amount to the initial drug amount ratio. It was calculated according to the following equation:

$$\text{Encapsulation efficiency} = \frac{Encapsulated\ drug\ amount}{Initial\ drug\ amount} \times 100$$

2.2.3. In vitro release:

In vitro release experiments were carried out for formulation CH8 (See Table 1). Nanoparticles suspension was centrifuged at 30000 rpm for 30 minutes. Collected nanoparticles were put in dissolution apparatus. The paddle method was used. Rotation speed was set at 75 rpm. Temperature was fixed at 37°C ± 0.5 °C. *In vitro* release was investigated, separately, at two different mediums: 900 ml of 0.1N HCl pH1.2 and 900ml of Phosphate buffer (PBS) pH6.8. At predetermined intervals, 10 ml of release medium were withdrawn and replaced by 10 ml of fresh medium to maintain the sink conditions. Released ALD amount was determined by the HPLC method as described above. Cumulative drug release was then calculated.

2.2.4. Statistical analysis:

All data in tables and figures were expressed as mean ± standard deviation. Statistical analysis was carried out using the one-way analysis of variance (ANOVA) and one-tailed unpaired Student's t tests. $p<0.05$ was chosen as criterion for statistical significance.

3. Result and discussion:

3.1.Nanoparticles characterization:

3.1.1. Particle size, zeta potential and morphology analysis:

Table 2 shows particle size and Zeta potential of the obtained nanoparticles. Obtained particle sizes were in the range of 91-175 nm. TEM images showed nanoparticles with spherical and regular form (See **Fig. 1.**). Generally, particles obtained with chitosan by ionic gelation are small and rarely exceed 250 nm [18]. For the same chitosan to TPP ratios, the increase of chitosan and TPP concentration from 1mg/ml to 2mg/ml resulted in a significant increase in particle size ($p<0.05$) (See **Fig. 2**). In fact, chitosan concentration is a critical parameter that monitors particle size. Chitosan concentrations above 2mg/ml resulted in particle aggregation and large particle formation. In fact, it was shown that final chitosan concentration (after TPP adding) must be below 1.5 mg/ml to form submicron particles and not large aggregates. It was explained that, when chitosan concentration is high, particle size increases because chitosan chains approach each other. Consequently, equilibrium between hydrogen bond attractions and electrostatic repulsions between chitosan nanoparticles is broken. This results in the formation of large micro-objects [19]. Variation of chitosan to TPP ratio resulted also, generally, in a significant variation in particle size. Particle size first decreased then increased when chitosan to TPP gradually increased from 2.5 to 5 for formulations CH1 to CH4. However, more pronounced and continuous size increase was noticed when chitosan to TPP ratio increased from formulation CH5 to CH8 ($p<0.05$). Those results are confirmed by other data reported in literature [20]. Obtained zeta potentials were all positive and they ranged from +21 to +27 mV (See **Fig. 3**). Positive Zeta values are explained by the positive charges of amino groups of chitosan which are present at particles surface. Ionic gelation is based on transformation of cationic chitosan to a gel following the addition of anionic TPP. Particles kept their positive charge despite TPP addition because chitosan molecular weight is higher than TPP molecular weight. At equal chitosan to TPP ratios, no significant variation of Zeta potential was noticed except between formulations CH2 and CH6 (ratio=3.33) ($p<0.05$).

Chitosan to TPP ratio did not exert a stable effect on zeta potential for formulation CH1 to CH4. However, continuous increase of Zeta potential was observed from formulation CH5 to CH8 when chitosan to TPP ratio increased. The same effect was described by de Pinho Neves et al. [17]. This could be explained by an increase of the positive charges provided by chitosan following increase of chitosan amount. Obtained zeta potential values ensured good stability for nanoparticles. No aggregation was observed even after storage for 3 months at ambient temperature. To have nanoparticles with good size uniformity, low molecular weight and small concentrations of chitosan should be used. The use of higher molecular weight resulted in particles aggregation and pronounced polydispersity (data not shown). It was shown that at same chitosan to TPP ratios, encapsulation efficiency increased significantly when chitosan and TPP concentrations increased. It seems that the increase of chitosan amount allowed entrapment of high ALD amounts thanks to more electrostatic interactions with much more positive charges from chitosan. In addition, large particles, generally, could encapsulate high active amounts as particle surface and volume got increased. At same chitosan and TPP concentrations, encapsulation efficiency was generally influenced by chitosan to TPP ratio. For both series, encapsulation efficiency first decreased then increased again (See **Fig. 4**). Formulation CH8 provided the highest value of encapsulation efficiency (70%). Compared to our previous study (Highest encapsulation efficiency is 34.31%), a remarkable enhancement in encapsulation efficiency was reached [9]. Obtained encapsulation efficiency is also higher than the value which was obtained by Cohen Sela et al. [21] and smaller than the value that was reported by Cohen Sela et al. [22]. However, our findings are interesting as we used less polymer amount which renders the drug loading more important. Accordingly, it is concluded that hydrophilic nature of chitosan and ALD, their opposite charges (positive charge for chitosan and negative charge for ALD) favored their interaction. This resulted in high active encapsulation efficiency. Actually, chitosan has been successfully used to encapsulate hydrophilic molecules. Encapsulated molecules ranged from small actives to macromolecules like DNA, RNA and proteins [23][24][25][26].

Table 2. Properties of prepared formulations

Formulation code	Size (nm)	Zeta (mV)	Encapsulation efficiency (%)
CH1	98±0.4	21±2.1	50.62±0.36
CH2	91±1.1	21±0.6	50.84±0.08
CH3	91±0.5	27±2	46.20±0.07
CH4	96±1.1	24±0.4	47.89±0.29
CH5	140±1.4	23±1.3	66.39±0.06
CH6	157±2	23±0.2	59.07±0.07
CH7	159±1.2	25±0.7	69.40±0.18
CH8	175±2.2	26±1.4	70.06±0.05

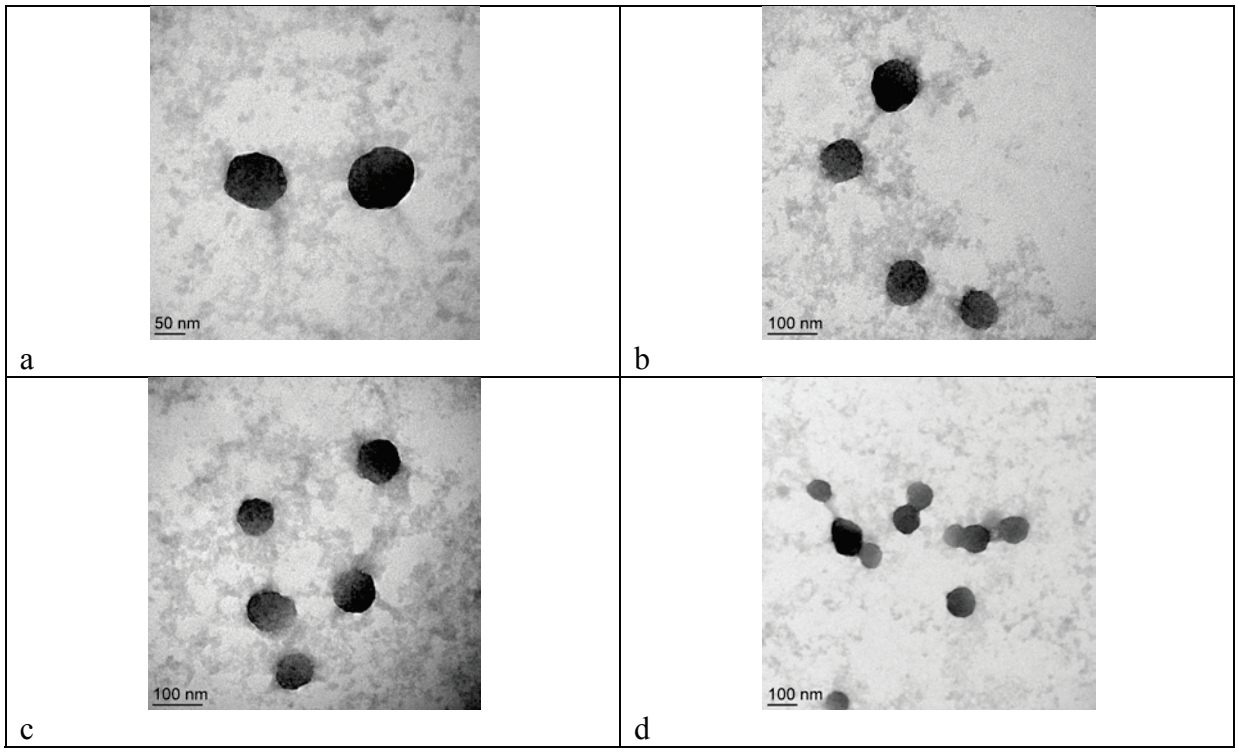


Fig.1. TEM images of chitosan nanoparticles

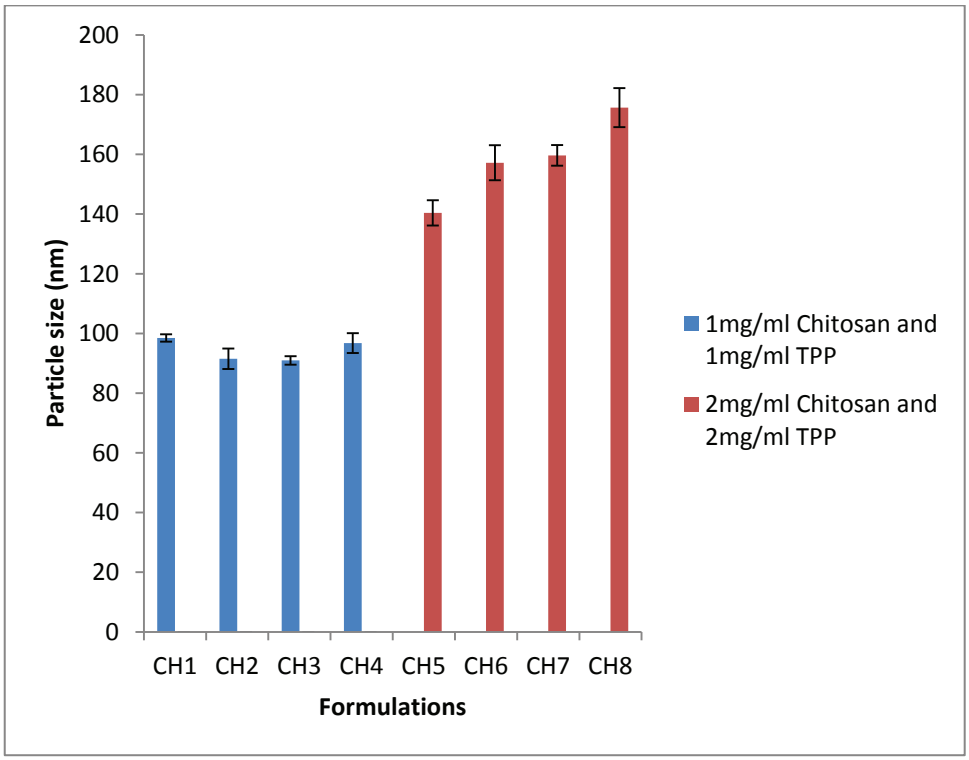


Fig. 2. Formulations versus hydrodynamic particle size

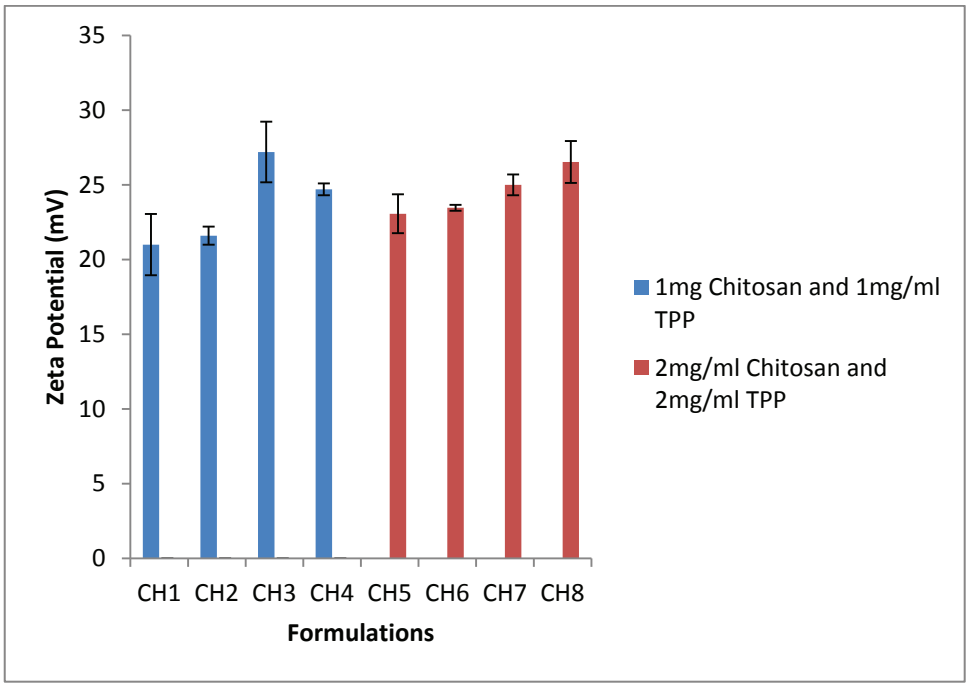


Fig. 3. Formulations versus zeta potential

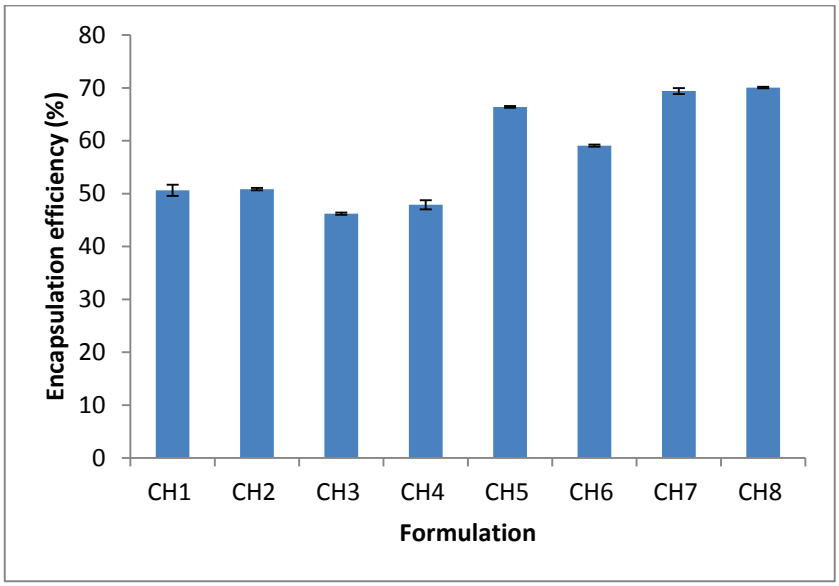


Fig. 4. Formulations versus encapsulation efficiency

3.1.2. FTIR analysis:

The infrared studies of chitosan and chitosan nanoparticles were performed to characterize the chemical structure of biopolymer and nanoparticles. **Fig. 5.** displays the FTIR spectrum of alendronate powder, chitosan powder, non-loaded and loaded nanoparticles. Alendronate spectrum presented strong bands on the region 1200-900 cm^{-1} that correspond to C-O and P=O stretches. Chitosan spectrum has characteristic peaks at 3321 cm^{-1} (OH and NH_2 stretching), 2866 cm^{-1}(CH stretching) and 1647cm^{-1} (amide I). For chitosan nanoparticles, new peaks appear at 1226 cm^{-1}(C-O-C stretch) and 1547 cm^{-1} (amide II) which resulted from the complex formation via electrostatic interaction between NH_3^+ groups of chitosan and phosphoric groups of TPP. The characteristic peak of alendronate in the region 900 cm^{-1} is also seen in nanoparticles loaded with alendronate and absent in blank nanoparticles. This result confirms that ALD is encapsulated inside nanoparticles [27].

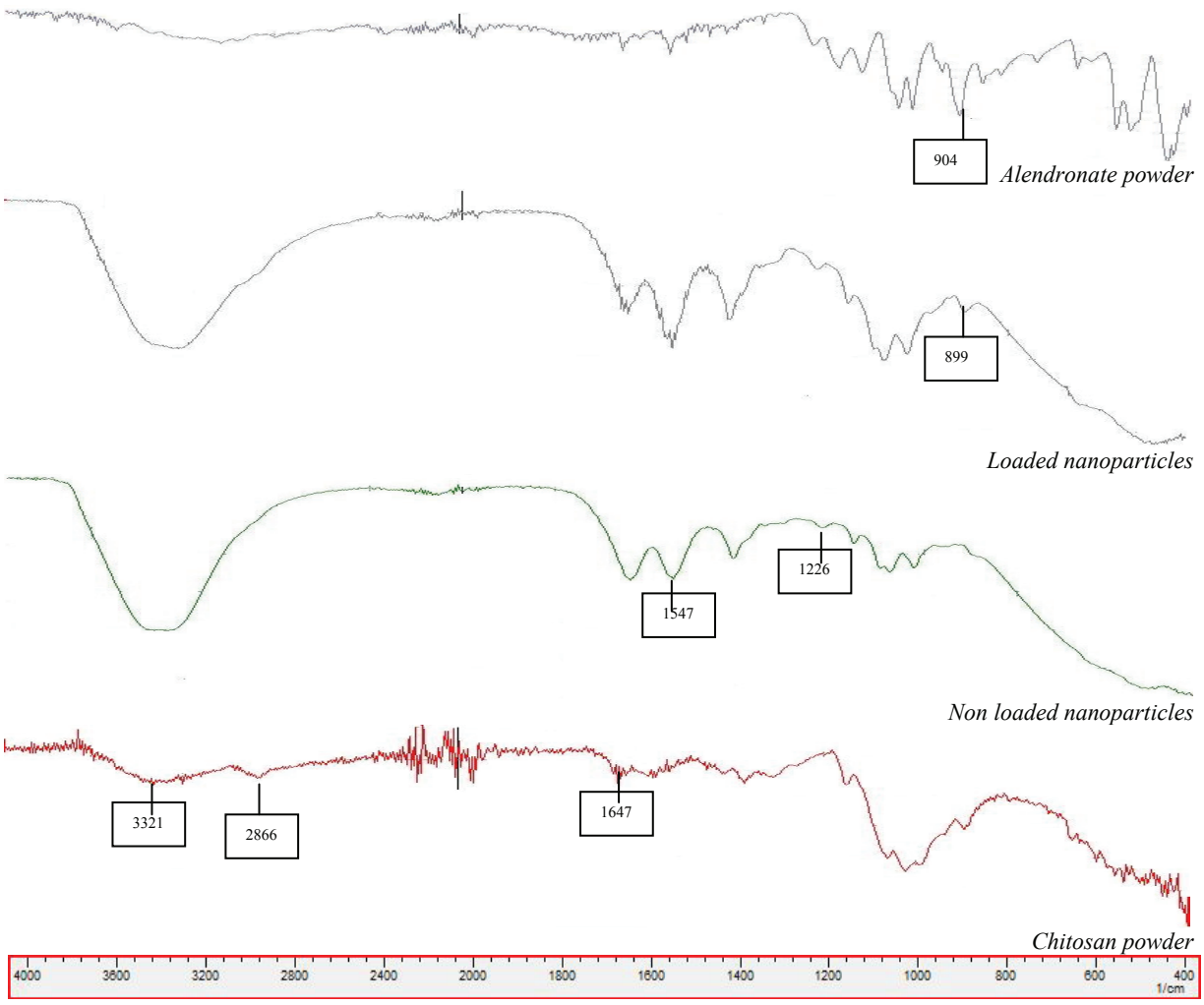


Fig. 5. FTIR analysis

3.2.In vitro release:

Obtained data from *in vitro* release studies were plotted in a curve which is shown by **Fig. 6.** Particles provided sustained release for ALD. It could be also concluded that release profile was different depending on the medium. In fact, ALD release in 0.1N HCl was faster than release obtained in PBS pH6.8. More than a half of the encapsulated ALD was released in 0.1N HCl in 30 min. At the same period, less than 18% of encapsulated ALD was released in PBS pH6.8 medium. Kinetic equilibrium in 0.1N HCl was reached in 60 min. By this time, more than 80% of encapsulated active was released. Released ALD increased then slightly to reach a maximum of cumulative release of 93%. However, for PBS medium, equilibrium was reached in 240 min. At that time, only less than 42% of encapsulated active was released. At the end of the study (360 min), only 42% of encapsulated active was released. These results could be explained by the properties of chitosan. It is well known that chitosan is only soluble

in acidic conditions. This resulted in a rapid release of the active at acidic 0.1N HCl. Conversely, poor solubility of chitosan in neutral conditions hampered the release of high ALD amounts from chitosan nanoparticles at pH6.8 [28]. However, both release kinetics presented globally similar profiles with a first burst release phase followed by a more prolonged release phase. Initial phase release could be linked to rapid hydration of nanoparticles due to the hydrophilic nature of chitosan. This phenomenon might also be due to the release of ALD which is adsorbed on nanoparticles surface [29]. In fact, "drug release" refers to the process in which drug molecules migrate from the initial position in the polymeric system to the polymer's outer surface and then to the release medium. This process is affected by multiple complex factors such as the physicochemical properties of the solutes (solubility, charges, interaction with matrix), the structural characteristics of the material system (structure, swelling, degradation), release environment (pH, temperature, ionic strength), and the possible interactions between these factors [30].

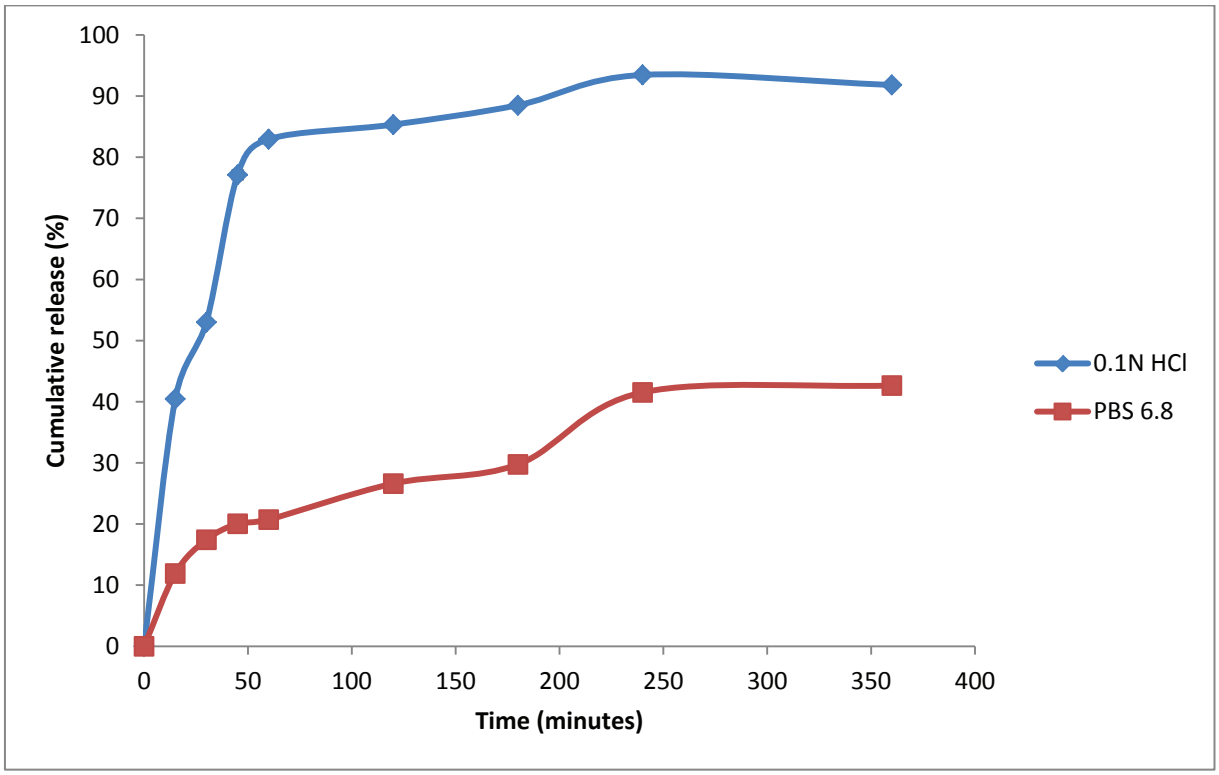


Fig. 6. In vitro release of alendronate from formulation CH8 in 0.1N HCL and PBS pH6.8

4. Conclusion:

Preparation of chitosan nanoparticles loaded with alendronate sodium by the ionic gelation technique was performed. Prepared nanoparticles presented different but submicronic sizes depending on operating conditions. Chitosan and TPP concentrations exerted always a significant effect on size and encapsulation efficiency values. An encapsulation efficiency around 70% was obtained. Nanoparticles released alendronate faster when they were put in 0.1N HCl than when they were introduced in PBS pH6.8. This implies that release profile of ALD from nanoparticles would be significantly different in the stomach and the intestine. *In vivo* studies will be soon carried out to assess the actual potential of the prepared particles regarding bioavailability and gastro-intestinal tolerance.

5. Acknowledgements:

We would like to acknowledge support from Tunisian Ministry Of Higher Education And Scientific Research by awarding a grant. We would like also to thank Ahmad Bitar and Géraldine Agusti for their help with the TEM analysis.

References:

[1] K. Miladi, S. Sfar, H. Fessi, A. Elaissari, Drug carriers in osteoporosis: preparation, drug encapsulation and applications, Int. J. Pharm. 445 (2013) 181–195.

[2] S. Mazzaferro, K. Bouchemal, A. Maksimenko, R. Skanji, P. Opolon, G. Ponchel, Reduced Intestinal Toxicity of Docetaxel Loaded Into Mucoadhesive Nanoparticles, in Mouse Xenograft Model, J. Colloid Sci. Biotechnol. 1 (2012) 210–217.

[3] V. Rosset, N. Ahmed, I. Zaanoun, B. Stella, H. Fessi, A. Elaissari, Elaboration of Argan Oil Nanocapsules Containing Naproxen for Cosmetic and Transdermal Local Application, J. Colloid Sci. Biotechnol. 1 (2012) 218–224.

[4] M.S. Khan, G.D. Vishakante, A. Bathool, Development and Characterization of Brimonidine Tartrate Loaded Eudragit Nanosuspensions for Ocular Drug Delivery, J. Colloid Sci. Biotechnol. 1 (2012) 122–128.

[5] H. Fessi, F. Puisieux, J.P. Devissaguet, N. Ammoury, S. Benita, Nanocapsule formation by interfacial polymer deposition following solvent displacement, Int. J. Pharm. 55 (1989) R1–R4.

[6] W. Hachicha, L. Kodjikian, H. Fessi, Preparation of vancomycin microparticles: Importance of preparation parameters, Int. J. Pharm. 324 (2006) 176–184.

[7] D. Quintanar-Guerrero, E. Allémann, H. Fessi, E. Doelker, Pseudolatex preparation using a novel emulsion-diffusion process involving direct displacement of partially water-miscible solvents by distillation, Int. J. Pharm. 188 (1999) 155–164.

[8] A. Doustgani, E.V. Farahani, M. Imani, A.H. Doulabi, Dexamethasone Sodium Phosphate Release from Chitosan Nanoparticles Prepared by Ionic Gelation Method, J. Colloid Sci. Biotechnol. 1 (2012) 42–50.

[9] K. Miladi, S. Sfar, H. Fessi, A. Elaissari, Encapsulation of alendronate sodium by nanoprecipitation and double emulsion: From preparation to in vitro studies, Ind. Crops Prod. (2015).

[10] C.M. Riley, T.W. Rosanske, S.R.R. Riley, Specification of Drug Substances and Products: Development and Validation of Analytical Methods, Newnes, 2013.

[11] S. Mao, W. Sun, T. Kissel, Chitosan-based formulations for delivery of DNA and siRNA, Adv. Drug Deliv. Rev. 62 (2010) 12–27.

[12] S.A. Agnihotri, N.N. Mallikarjuna, T.M. Aminabhavi, Recent advances on chitosan-based micro- and nanoparticles in drug delivery, J. Control. Release Off. J. Control. Release Soc. 100 (2004) 5–28.

[13] A. Mahapatro, D.K. Singh, Biodegradable nanoparticles are excellent vehicle for site directed in-vivo delivery of drugs and vaccines, J. Nanobiotechnology. 9 (2011) 55.

[14] L. Casettari, L. Illum, Chitosan in nasal delivery systems for therapeutic drugs, J. Control. Release Off. J. Control. Release Soc. 190 (2014) 189–200.

[15] M.-C. Chen, F.-L. Mi, Z.-X. Liao, C.-W. Hsiao, K. Sonaje, M.-F. Chung, et al., Recent advances in chitosan-based nanoparticles for oral delivery of macromolecules, Adv. Drug Deliv. Rev. 65 (2013) 865–879.

[16] S. Wadhwa, R. Paliwal, S.R. Paliwal, S.P. Vyas, Chitosan and its role in ocular therapeutics, Mini Rev. Med. Chem. 9 (2009) 1639–1647.

[17] C.A. Silva, T.M. Nobre, F.J. Pavinatto, O.N. Oliveira Jr., Interaction of chitosan and mucin in a biomembrane model environment, J. Colloid Interface Sci. 376 (2012) 289–295.

[18] L. Bugnicourt, P. Alcouffe, C. Ladavière, Elaboration of chitosan nanoparticles: Favorable impact of a mild thermal treatment to obtain finely divided, spherical, and colloidally stable objects, Colloids Surf. Physicochem. Eng. Asp. 457 (2014) 476–486.

[19] W. Fan, W. Yan, Z. Xu, H. Ni, Formation mechanism of monodisperse, low molecular weight chitosan nanoparticles by ionic gelation technique, Colloids Surf. B Biointerfaces. 90 (2012) 21–27.

[20] A.L. de Pinho Neves, C.C. Milioli, L. Müller, H.G. Riella, N.C. Kuhnen, H.K. Stulzer, Factorial design as tool in chitosan nanoparticles development by ionic gelation technique, Colloids Surf. Physicochem. Eng. Asp. 445 (2014) 34–39.

[21] E. Cohen-Sela, O. Rosenzweig, J. Gao, H. Epstein, I. Gati, R. Reich, et al., Alendronate-loaded nanoparticles deplete monocytes and attenuate restenosis, J. Controlled Release. 113 (2006) 23–30.

[22] E. Cohen-Sela, M. Chorny, N. Koroukhov, H.D. Danenberg, G. Golomb, A new double emulsion solvent diffusion technique for encapsulating hydrophilic molecules in PLGA nanoparticles, J. Control. Release Off. J. Control. Release Soc. 133 (2009) 90–95.

[23] N.F.S. de Melo, E.V.R. Campos, E. de Paula, A.H. Rosa, L.F. Fraceto, Factorial Design and Characterization Studies for Articaine Hydrochloride Loaded Alginate/Chitosan Nanoparticles, J. Colloid Sci. Biotechnol. 2 (2013) 146–152.

[24] N. Csaba, M. Köping-Höggård, M.J. Alonso, Ionically crosslinked chitosan/tripolyphosphate nanoparticles for oligonucleotide and plasmid DNA delivery, Int. J. Pharm. 382 (2009) 205–214.

[25] K.A. Howard, S.R. Paludan, M.A. Behlke, F. Besenbacher, B. Deleuran, J. Kjems, Chitosan/siRNA nanoparticle-mediated TNF-alpha knockdown in peritoneal macrophages for anti-inflammatory treatment in a murine arthritis model, Mol. Ther. J. Am. Soc. Gene Ther. 17 (2009) 162–168.

[26] M.R. Avadi, A.M.M. Sadeghi, N. Mohammadpour, S. Abedin, F. Atyabi, R. Dinarvand, et al., Preparation and characterization of insulin nanoparticles using chitosan and Arabic gum with ionic gelation method, Nanomedicine Nanotechnol. Biol. Med. 6 (2010) 58–63.

[27] S.F. Hosseini, M. Zandi, M. Rezaei, F. Farahmandghavi, Two-step method for encapsulation of oregano essential oil in chitosan nanoparticles: Preparation, characterization and in vitro release study, Carbohydr. Polym. 95 (2013) 50–56.

[28] S.K. Motwani, S. Chopra, S. Talegaonkar, K. Kohli, F.J. Ahmad, R.K. Khar, Chitosan-sodium alginate nanoparticles as submicroscopic reservoirs for ocular delivery: formulation, optimisation and in vitro characterisation, Eur. J. Pharm. Biopharm. Off. J. Arbeitsgemeinschaft Für Pharm. Verfahrenstechnik EV. 68 (2008) 513–525.

[29] S.P. Egusquiaguirre, C. Manguán-García, L. Pintado-Berninches, L. Iarriccio, D. Carbajo, F. Albericio, et al., Development of surface modified biodegradable polymeric nanoparticles to deliver GSE24.2 peptide to cells: A promising approach for the treatment of defective telomerase disorders, Eur. J. Pharm. Biopharm. Off. J. Arbeitsgemeinschaft Pharm. Verfahrenstechnik EV. (2015).

[30] Y. Fu, W.J. Kao, Drug Release Kinetics and Transport Mechanisms of Non-degradable and Degradable Polymeric Delivery Systems, Expert Opin. Drug Deliv. 7 (2010) 429–444.

La tolérance et la pharmacocinétique des nanoparticules contenant l'alendronate de sodium

La troisième partie expérimentale consiste en une étude *in vivo* des effets indésirables et de la biodisponibilité des nanoparticules de chitosane qui ont été préparées au cours de la l'étude précédente. Cette partie comprend deux études : une étude de tolérance et une étude pharmacocinétique. L'étude a été réalisée chez des rats Wistar mâles pesant entre 280 et 350g. La préparation de nanoparticules administrée chez les rats correspond à la formule CH8. L'étude de tolérance gastrique a été réalisée en deux doses différentes d'alendronate : une forte dose (80 mg/Kg/j) et une faible dose (30 mg/Kg/j). A chaque fois, l'étude a été réalisée sur trois groupes de rats. Un groupe « témoin » a reçu des nanoparticules vides. Un groupe « référence » a reçu une solution d'alendronate et un groupe « nanoparticules » a reçu les nanoparticules chargées en alendronate. Lors de la première procédure, on a procédé à l'administration d'une dose d'alendronate équivalente à 80 mg/Kg/j pendant 4 jours par gavage. Cinq mortalités ont été observées dans le groupe « référence » alors qu'un seul rat est mort dans le groupe « nanoparticules ». En revanche, aucune mortalité n'a été enregistrée dans le groupe « témoin ». Ceci suggère l'existence d'une action protectrice conférée par les nanoparticules contre les effets toxiques de l'alendronate. En effet, on a observé chez les rats morts un enflement au niveau de l'estomac et des intestins. Cet enflement a engendré une compression sur les poumons ce qui a provoqué à un certain moment un grand gène respiratoire chez le rats. La deuxième procédure a consisté en l'administration d'une dose plus faible équivalente à 30 mg/Kg/j pendant 15 jours. Au cours de cette étude, 2 mortalités ont été enregistrées dans le groupe « référence » et une mortalité a été également observée dans le groupe « nanoparticules ». Un autre rat du groupe « nanoparticules » a été mis à mort pour éviter une grande souffrance de l'animal. Tous les rats du groupe « témoin » sont restés vivants. A la fin de chaque étude de tolérance, les rats ont été mis à mort. Les œsophages, les estomacs ainsi qu'une partie des intestins ont été prélevés pour subir par la suite une étude anatomopathologique à la recherche de lésions histologiques. L'étude pharmacocinétique a été réalisée sur deux groupes. Un groupe « témoin » a reçu une solution d'alendronate alors que le groupe « nanoparticules » a reçu les nanoparticules chargées en alendronate. La dose administrée d'alendronate chez les deux groupes a été de 60 mg/Kg. Les deux préparations ont été administrées par voie orale par gavage. Un volume sanguin constant de 0,3 ml a été prélevé à partir de la veine caudale à des intervalles prédéterminés sur 7 heures. Pour tous les prélèvements sanguins, le plasma a été séparé puis une extraction en phase solide a été réalisée pour séparer l'actif. Le dosage de l'alendonate a été réalisé par chromtographie

liquide à haute performance (HPLC) avec une détection fluorimétrique. Nous avons réussi à améliorer la résolution des pics de l'alendronate à la HPLC mais d'autres études sont encore indispensables pour améliorer la linéarité de la courbe d'étalonnage et la séparation de l'alendronate. Les voies d'optimisation concernent toutes les étapes de l'étude à savoir : (1) le prélèvement sanguin, (2) la séparation du plasma, (3) la déprotéinisation, (4) l'extraction en phase solide, (5) la dérivatisation de l'alendronate et (6) la méthode HPLC proprement dite. Les voies qui sont étudiées sont soit une optimisation de la technique de dosage par HPLC couplée à la fluorimétrie ou bien un changement de la technique de dosage en utilisant la méthode de chromatographie couplée à la spectrométrie de masse. Une optimisation de la technique d'extraction est aussi à envisager.

Une étude *in vivo* de la tolérance et de la biodisponibilité a été réalisée sur la formule CH8 préparée au cours de la partie expérimentale précédente.

1. Etude de tolérance:

Cette étude a été réalisée à deux doses différentes 80 mg/Kg/j pendant 4 jours et 30 mg/Kg/j pendant 2 semaines. Trois groupes de rats comportant chacun 6 rats ont été constitués : un groupe « Nanoparticules » a reçu les nanoparticules chargées en alendronate de sodium, un groupe « Référence » chez lequel nous avons administré une solution d'alendronate et enfin, un groupe « Témoin » qui a reçu les nanoparticules vides.

1.1.Etude réalisée à forte dose (80 mg/Kg/j) :

Groupe	Mortalités	Ulcérations
Références	5	5 (estomac et intestin grêle)
Nanoparticules	1	1 (estomac)
Témoin	0	0

Les résulats obtenus sont en faveur d'une action protectrice conférée par les nanoparticules contre les effets indésirables de l'alendronate. Les mortalités observées sont dues à des ulcérations au niveau digestif qui ont entrainé un enflement au niveau gatrointestinal. Ce dernier a engendré une compression sur les poumons et un grand gène respiratoire chez les animaux.

1.2.Etude réalisée à faible dose (30 mg/Kg/j) :

Groupe	Mortalités	Ulcérations (U) ou inflammations (I)
Références	2	1U (estomac) + 3I (œsophage, estomac et intestin grêle)
Nanoparticules	1	2I (estomac)
Témoin	0	0

Les résultats obtenus mettent en évidence un effet protecteur des nanoparticules. Cependant, nous constatons qu'un tel effet est plus remarquable à forte dose.

2. Etude pharmacocinétique :

Les prélèvements sanguins ont été réalisés pour tous les groupes. La séparation du plasma et l'extraction de l'alendronate de sodium ont été aussi effectuées. Le dosage de l'alendonate de sodium a été réalisé après dérivatisation par HPLC avec détection fluorimétrique. La méthode de dosage est en cours d'optimisation pour améliorer la résolution des pics et la linéarité de la courbe d'étalonnage.

Discussion générale et conclusion

Le but de notre projet était de présenter une nouvelle alternative thérapeutique en adoptant l'approche d'encapsulation de la molécule active dans des nanoparticules. Notre étude bibliographique a démontré que le traitement pharmacologique de l'ostéoporose, en général, présente plusieurs limites. L'encapsulation des substances actives anti-ostéoporotiques a permis d'apporter plusieurs bénéfices thérapeutiques par rapport aux formes conventionnelles. Les techniques qui ont été les plus utilisées sont : l'émulsion évaporation du solvant, le spray-drying, la polymérisation en émulsion, la gélification ionique, l'hydratation du film lipidique, l'émulsion diffusion du solvant et l'adsorption des molécules actives sur des particules. La plupart des formes préparées étaient des microparticules. Les avantages qui ont été décrits sont : la protection de la molécule active contre la dégradation (ce qui permet de prolonger sa demi-vie), la libération prolongée, la diminution des effets indésirables, l'augmentation de l'absorption à travers des membranes biologiques et un meilleur ciblage du tissu osseux. Ceci a été prouvé par des études *in vitro* et *in vivo*. Les paramètres expérimentaux ont joué aussi un rôle important sur le comportement *in vitro* et *in vivo* des particules préparées.

Les techniques utilisées pour l'encapsulation sont multiples mais le principe général est de précipiter un polymère qui se trouve initialement en solution. Ceci peut avoir lieu après l'addition d'un non solvant ou l'ajout d'un sel. La plupart des méthodes d'encapsulation utilisent des solvants organiques qui seront évaporés à une étape finale. Cependant, certaines méthodes présentent l'avantage de ne pas utiliser des solvants organiques comme la technique de gélification ionique ou les techniques qui utilisent les fluides supercritiques. Les polymères les plus utilisés pour des applications *in vivo* sont biodégradables et biocompatibles. Les propriétés d'un polymère déterminé peuvent avoir une répercussion sur le comportement *in vivo*. Par exemple, le chitosane présente des charges positives ce qui lui confère des propriétés mucoadhésives. Les travaux de recherche les plus innovants utilisent un ciblage des particules préparées par un ligand qui va reconnaitre spécifiquement une cellule ou un tissu particulier. Ceci permet d'augmenter l'efficacité et d'améliorer la tolérance. On constate aussi une tendance vers le développement de vecteurs intelligents grâce aux « matériaux stimulables ». Ces derniers libèrent la substance active suite à une variation de température (ce qui est intéressant en cas d'infections) ou de pH (exemple du diabète). Plusieurs efforts ont été fournis également pour améliorer les techniques conventionnelles. C'est l'exemple de la nanoprécipitation avec le développement des techniques d'émulsification par membrane, de la microfluidique et de la nanoprécipitation flash. Ces techniques permettent de diminuer les

volumes de solvants organiques utilisées. Elles permettent aussi d'améliorer la reproductibilité et d'assurer une meilleure transposition d'échelle.

Tout au long de notre projet, nous avons réussi à développer, préparer et caractériser des nanoparticules polymériques chargées en alendronate de sodium. L'encapsulation des molécules hydrophiles comme l'alendronate de sodium représente un grand défi vu le nombre limité de techniques permettant leur encapsulation. Deux méthodes de préparation de nanoparticules ont été utilisées : l'émulsion double et la nanoprécipitation. Les particules ont été caractérisées en étudiant la taille et la forme (par diffusion de la lumière dynamique et microscopie électronique à transmission), le potentiel Zêta et l'état physique du polymère et de la substance active (par calorimétrie différentielle à balayage et par spectroscopie infrarouge à transformée de Fourier). Le pourcentage d'encapsulation et le taux de chargement en actif ont été déterminés par spectrophotométrie ultraviolet puis par chromatographie liquide à haute performance. Les nanoparticules à base de poly-ε-caprolactone ont permis d'obtenir un pourcentage d'encapsulation maximal de 34% dans le cas de la double émulsion et de 18% pour la nanoprécipitation. L'étude systématique a, d'autre part, mis en évidence l'influence de paramètres expérimentaux sur la taille et l'efficacité d'encapsulation. La meilleure encapsulation obtenue pour l'émulsion double confirme que cette technique est mieux adaptée pour l'encapsulation de substances actives hydrophiles. Pour les deux méthodes, on constate qu'une augmentation de la quantité ou du poids moléculaire du polymère entraine une augmentation de la taille des nanoparticules. Le ratio phase organique/phase aqueuse a aussi exercé un effet significatif sur la taille des nanoparticules. En effet, une augmentation du volume de la phase aqueuse a entrainé une diminution de la taille des nanoparticules. Le ratio actif/polymère et le poids moléculaire du polymère n'ont pas significativement influé le potentiel Zêta. L'augmentation du volume de la phase aqueuse s'est traduite par une diminution du pourcentage d'encapsulation.

En revanche, l'utilisation des solvants toxiques comme l'acétone et le dichlorométhane ainsi que le profil de libération fortement prolongé représentaient des limites pour une application *in vivo*. Vu ces premiers résultats, notre intérêt s'est focalisé, par la suite, sur la préparation de nanoparticules à base de chitosane. Celui-ci est un polymère naturel hydrophile ayant des propriétés intéressantes en encapsulation. En effet, les charges positives que porte le chitosane permettent une forte interaction électrostatique avec la muqueuse gastro-intestinale ce qui améliore le passage des substances actives à travers les membranes biologiques et augmente donc leurs biodisponibilités. La technique utilisée pour la préparation des nanoparticules est la

gélification ionique. Cette méthode est simple et ne nécessite pas l'utilisation de solvants organiques. Elle se base sur le passage d'un polymère dissous de la forme liquide à la forme gel. Dans notre cas, ce changement d'état est du à une interaction électrostatique entre le chitosane (chargé positivement) et le tripolyphosphate de sodium (chargé négativement). La taille des particules obtenues a varié entre 91 et 175 nm. Les images de microscopie électronique de transmission ont montré des nanoparticules de forme sphérique régulière. Le potentiel Zêta a été positif et les valeurs étaient dans l'intervalle 21-27 mV. Ces valeurs positives s'expliquent par la présence de groupements amines quaternaires dans la structure du chitosane. L'étude systématique a montré qu'une augmentation de la concentration du chitosane ou du tripolyphosphate de sodium entraine une augmentation de la taille des particules. Dans les mêmes conditions, une augmentation du potentiel Zêta a été obtenue. Le ratio chitosane/tripolyphosphate a généralement entrainé une augmentation significative de la taille des particules. L'effet du ratio chitosane/tripolyphosphate était variable. Lorsque la concentration du chitosane et tripolyphosphate était de 1mg/ml, l'effet du changement du ratio était variable. Par contre, à une concentration de chitosane et de tripolyphosphate égale à 2mg/ml, une augmentation constante du potentiel Zêta a été observée. Ceci peut être expliqué par une augmentation des charges positives suite à l'addition du chitosane. A des ratios chitosane/tripolyphosphate égaux, une augmentation de la concentration du chitosane et du tripolyphosphate a entrainé une augmentation du pourcentage d'encapsulation. L'augmentation de la concentration du chitosane (chargé positivement) pourrait être à l'origine d'une interaction plus forte avec l'alendronate (chargé négativement). Une tendance générale d'augmentation de l'efficacité d'encapsulation a été aussi observée. La valeur maximale d'efficacité d'encapsulation obtenue a été 70%. De ce fait, une amélioration nette de l'efficacité d'encapsulation a été obtenue par rapport à la première étude où on a obtenu un maximum de 34%. Cette amélioration peut être expliquée par une plus forte interaction substance active-polymère. En effet, l'alendronate et le chitosane sont tous les deux hydrophiles et ils possèdent également des charges opposées. L'étude de la libération *in vitro* a permis de déceler une différence de libération qui dépend du pH du milieu. En effet, la libération de l'alendronate a été plus rapide en milieu HCl 0,1N qu'en milieu tampon phosphate pH6,8. Ceci s'explique par la meilleure solubilité du chitosane à pH acide. Cependant, le profil général de libération a été similaire avec une phase de libération rapide qui est suivie par une phase de libération plus lente. La libération de l'alendronate à partir des nanoparticules de chitosane a été plus rapide que celle à partir des nanoparticules ce qui sera plus adapté à une administration *in vivo*. Cette amélioration est due à l'hydrophilie du

chitosane qui présente plus d'affinité pour le milieu de dissolution que la poly-ε-caprolactone hydrophobe. Nous avons ainsi réussi à améliorer l'efficacité d'encapsulation et à obtenir un profil de libération de la substance active *in vitro* plus adéquat.

L'encapsulation a été présentée comme une excellente approche pour améliorer l'efficacité des traitements. L'amélioration du profil biopharmaceutique de substances actives a aussi été confirmée. Dans notre cas, nous nous sommes intéressés aux formes nanoparticules qui n'ont pas été largement développées pour l'ostéoporose. On s'intéresse aussi à l'alendronate vu la place importante qu'il occupe dans la thérapie anti-ostéoporose. En effet, il est efficace et prescrit en première intention. L'encapsulation des molécules hydrophiles présente un grand défi vu le nombre réduit de techniques qui permettent leur encapsulation. Nous avons réussi à préparer des nanoparticules polymériques à base d'alendronate de sodium, de les caractériser et étudier leurs profils de libération *in vitro*. Nous avons aussi réussir à améliorer le pourcentage d'encapsulation de la molécule active ainsi que le profil de libération *in vitro*.

Grâce aux études réalisées on a pu déterminer et maitriser les facteurs expérimentaux qui affectent les propriétés des nanoparticules préparées par trois méthodes : la nanoprécipitation, l'émulsion double et la gélification ionique. Les études bibliographiques et pratiques ont permis de mieux connaitre les mécanismes de formation des nanoparticules ainsi que de trouver une explication aux variations des propriétés des nanoparticules en fonction des conditions opératoires. Le dosage de la substance active a été maitrisé par deux techniques à savoir, la spectrophotométrie ultraviolet et la chromatographie liquide à haute performance. Nous avons pu analyser les données de libération *in vitro* et prendre connaissance des paramètres qui conditionnent la diffusion de l'alendronate. Ainsi, le caractère hydrophile du chitosane a permis d'assurer une libération plus rapide de l'alendronate par rapport à la poly-ε-caprolactone au caractère plutôt lipophile. Nous avons pu aussi mener une étude *in vivo* chez le rat pour étudier la tolérance la biodisponibilité. Au cours de l'étude de tolérance, un nombre peu élevé de mortalités a été obtenu chez les groupes ayant reçu les nanoparticules par rapport aux groupes qui ont reçu la solution d'alendronate. Ceci suppose qu'une action protectrice a été exerecée par notre préparation. Pour l'étude pharmacocinétique, une optimisation de la méthode du dosage plasmatique est indispensable pour l'interprétation des résultats.

Perspectives

Les études *in vivo* réalisées chez les rats nécessitent une optimisation de la technique d'extraction et du dosage plasmatique de l'alendronate de sodium. D'autres études *in vivo* peuvent être réalisées pour conduire une analyse comparative entre les formes nanoparticules et la forme commercialisée en comprimés (Fosamax®) qui servira de référence. Il est également intéressant de réaliser une étude à l'aide d'un modèle pharmacodynamique qui confirmera l'efficacité *in vivo*. Comme modèle, on peut évoquer les rates ovariectomisées. D'autres voies de recherche sont possibles telles que l'utilisation de ciblage par des molécules qui ont une forte affinité pour le tissu osseux comme les tétracyclines. L'approche théranostique peut être aussi intéressante par l'utilisation de particules magnétiques contenant de l'alendronate de sodium. De telles particules peuvent être une fois administrées dans l'organisme détectées par imagerie par résonnance magnétique. Elles vont ainsi agir à la fois comme agent de diagnostic par imagerie et comme traitement pharmacologique.

Annexes

Les formes nanoparticulaires ont été énormément étudiées au cours de ces dernières décennies. En effet, de telles formes présentent plusieurs avantages par rapport aux formes conventionnelles grâce à un meilleur passage à travers les membranes. Les techniques utilisées pour la fabrication des particules submicroniques sont nombreuses. Cependant, la nanoprécipitation (appelée aussi technique de déplacement du solvant) reste la technique la plus simple et la plus reproductible. La nanoprécipitation est très utilisée pour l'obtention de nanoparticules qui sont destinées à des applications biomédicales. La méthode est essentiellement utilisée pour l'encapsulation de molécules hydrophobes. On constate, pourtant, que plusieurs molécules actives hydrophiles ont été encapsulées avec succès via ce procédé. Les différentes approches qui ont été utilisées pour permettre l'encapsulation des molécules hydrophiles ont été exposées. Plusieurs conditions opératoires (quantité du polymère et son poids moléculaire, quantité d'agent stabilisant, ratio phase organique/phase aqueuse et vitesse d'agitation) peuvent influencer significativement les propriétés des particules obtenues voire même leur libération de l'actif encapsulé et leur efficacité *in vivo*. Plusieurs efforts ont été réalisés pour améliorer non seulement la reproductibilité de la nanoprécipitation mais aussi les propriétés physicochimiques et colloïdales des dispersions. D'autres techniques plus sophistiquées ont été également décrites telles que l'utilisation de l'agitateur en forme de « T », l'émulsification par membrane, les dispositifs microfluidiques et la nanoprécipitation flash. Ces techniques assurent une meilleure transposition d'échelle et une meilleure reproductibilité du processus de la nanoprécipitation. Par exemple, la nanoprécipitation flash permet de contrôler d'une façon plus efficace les propriétés des nanoparticules obtenues. Les avantages des nanoparticules préparées par nanoprécipitation ont été confirmés par de nombreuses études *in vivo*. Ils consistent en une amélioration de la biodisponibilité, un meilleur ciblage, une meilleure tolérance, une libération prolongée ou un meilleur passage à travers les membranes biologiques. Les études rapportées permettent aussi de conclure que la principale application *in vivo* des nanoparticules préparées par la nanoprécipitation est la thérapie anticancéreuse.

La nanoprécipitation offre plusieurs avantages par rapport aux autres techniques d'encapsulation. En fait, la technique est simple, ne nécessite pas l'utilisation de grand volumes de solvants organique. Une simple agitation magnétique suffit pour la formation des particules qui présentent une répartition de taille homogène. Deux phases miscibles sont utilisées : une phase organique et une phase aqueuse. Il a été démontré que le mécanisme de

formation des nanoparticules comprend trois phases : nucléation, croissance et agrégation. La supersaturation est présentée comme la force motrice de tous ces phénomènes. Elle est définie par le ratio de la concentration du polymère sur la solubilité du polymère dans le solvant organique. La dynamique des fluides et l'agitation des phases jouent aussi un rôle important. En effet, elle influe sur la supersaturation et, vu la rapidité du process de formation des particules, elle détermine aussi le taux de nucléation. Par conséquence, une faible agitation donne une faible quantité de grosses nanoparticules (un taux de nucléation bas) alors qu'une bonne agitation donne des taux de nucléation élevés et donc, une grande population de petites nanoparticules. La nanoprécipitation est principalement utilisée pour l'encapsulation de molécules hydrophobes ou amphiphiles. Cependant, plusieurs études ont décrit l'utilisation avec succès de la méthode pour l'encapsulation des molécules hydrophiles. Dans ce dernier cas, trois approches ont été utilisées : la dissolution de la substance active dans la phase aqueuse, l'utilisation d'un cosolvant ou la dissolution d'une faible quantité de la molécule active dans la phase organique. La composition de la phase organique est variable mais les solvants les plus utilisés sont l'acétone et l'éthanol. La phase aqueuse est généralement l'eau ou une solution aqueuse d'un stabilisant hydrophile comme le Pluronic® F68 ou l'alcool polyvinylique. Plusieurs polymères ont été utilisés pour la nanoprécipitation mais ce sont surtout les polyesters biodégradables qui sont utilisés et principalement, l'acide poly(lactique-co-glycolique). Plusieurs paramètres expérimentaux contrôlent les propriétés des particules obtenus. Une augmentation de la quantité du polymère entraine, généralement, une augmentation de la taille et du pourcentage d'encapsulation. Une augmentation du poids moléculaire se traduit par des effets variables sur la taille des nanoparticules. L'effet de l'augmentation de la quantité du stabilisant sur la taille est variable mais l'effet le plus observé est une diminution de la taille des particules. D'autre part, une diminution du rapport phase organique/phase aqueuse (ou autrement une augmentation du volume de la phase aqueuse) a entrainé une diminution de la taille des nanoparticules. Il a été aussi démontré que la nature des deux phases ainsi que leur ordre d'addition peut influer sur la taille des particules. En effet, des études ont montré une variation de la taille des particules en fonction de la nature phase organique. Cet effet serait imputable soit à une variation de viscosité ou de polarité. Dans certains cas, une variation du pH de la phase aqueuse a induit un effet sur le pourcentage d'encapsulation en modifiant la solubilité de la substance active. La vitesse d'agitation joue aussi un rôle important. Son augmentation se traduit souvent par une diminution de la taille des nanoparticules. Plusieurs approches innovantes reposant sur le principe de la nanoprécipitation ont été développées. Le but est d'améliorer la reproductibilité

et la possibilité de transposition d'échelle de la technique conventionnelle. Parmi ces approches, on cite l'émulsification par membrane, la microfluidique et la nanoprécipitation flash.

La technique de contacteur à membrane assure une bonne transposition d'échelle du fait qu'elle permet d'obtenir de grands volumes de suspension de nanoparticules. Dans cette technique, la phase organique (dispersée) passe à travers des petits pores. La phase aqueuse, quant à elle, passe tangentiellement. Ceci entraine une précipitation des gouttelettes de la phase organique contenant le polymère sous forme de particules. Cette technique a été appliquée pour encapsuler la vitamine E dans des nanoparticules de poly-ε-caprolactone. Une autre technique, la microfluidique, trouve son intérêt dans le fait qu'elle améliore énormément l'efficacité d'agitation des deux phases. Il s'agit généralement de micro-mélangeurs qui permettent de donner des nanoparticules plus petites que celles obtenues par la technique conventionnelle en utilisant moins de non-solvant. Ces micro-agitateurs permettent aussi de précipiter sous forme de nanoparticules des solutions de polymère à des concentrations supérieures à 5 % m/v ce qui est impossible à réaliser avec la méthode conventionnelle. En effet, celle-ci donne, dans ce cas, une population polydisperse. De même, la technique de nanoprécipitation flash a été développée pour améliorer les conditions d'agitation. Il s'agit de micro-agitateurs à entrées multiples qui permettent d'avoir des populations de particules monodisperses avec un bon pourcentage d'encapsulation de molécules actives. Via cette technique, la nanoprécipitation a lieu dans un temps beaucoup plus court que la technique conventionnelle.

Les particules préparées par nanoprécipitation ont été utilisées pour plusieurs applications *in vivo*. Les principales indications sont le traitement des cancers que ce soit par voie intraveineuse ou locale et le traitement des troubles oculaires.

Plusieurs substances actives hydrophobes ou hydrophiles présentent des problèmes de biodisponibilité, de stabilité ou de gout désagréable. L'encapsulation de telles molécules dans des nanoparticules est une approche intéressante pour pallier à ces inconvénients. Ainsi, une amélioration de l'efficacité du traitement et de la compliance des patients. La nanoprécipitation est une technique simple et reproductible qui est largement utilisée pour la préparation de nanoparticules destinées à des applications biomédicales. Les conditions opératoires ont une influence sur les propriétés des nanoparticules. Leur contrôle permet de maitriser la taille et le pourcentage d'encapsulation. Certains chercheurs ont utilisé la méthode

conventionnelle alors que d'autres ont choisi des méthodes plus innovantes pour améliorer la transposition d'échelle, la reproductibilité et diminuer l'utilisation de solvants organiques. Ainsi, la technologie de membrane, la microfluidique et la nanoprécipitation flash ont été utilisées avec succès. Les avantages des nanoparticules préparées par nanoprécipitation dans le domaine biomédical ont été confirmés par de nombreuses études *in vivo*. Parmi les avantages obtenus, on cite une amélioration de la biodisponibilité, un meilleur ciblage et tolérance, une libération prolongé et une amélioration de l'absorption de la substance active par les membranes biologiques.

Nanoprecipitation process: From particles preparation to *in vivo* applications

K. Miladi[a,b], S. Sfar[b], H. Fessi[a], A. Elaissari[a]*
[a] Université de Lyon, F- 69622, Lyon, France; Université Lyon 1, Villeurbanne, CNRS, UMR 5007, Laboratoire d'Automatique et de Génie des Procédés, LAGEP-CPE-308G, 43 bd. du 11 Nov.1918, F-69622, Villeurbanne, France.
[b] Université de Monastir, Laboratoire de pharmacie galénique, Rue Avicenne, 5000 Monastir, Tunisia

* Corresponding author: Phone: +33-472431841, Fax: +33-472431682

E-mail: elaissari@lagep.univ-lyon1.fr and fessi@lagep.univ-lyon1.fr

Abstract

Nanoparticles have been widely prepared during these last decades. In fact, encapsulation could provide several advantages over conventional pharmaceutical forms (Miladi et al. 2013)(Campos et al. 2013)(Grando et al. 2013)(de Melo et al. 2013)(Mazzaferro et al. 2012)(Lira et al. 2013)(Wang et al. 2012). Although, several techniques have been used for the preparation of submicron particles from preformed polymers, nanoprecipitation is regarded as a quite simple and reproducible technique that allows the obtaining of submicron sized polymer particles. Additionally, many research works have focused on the enhancement of the reproducibility of the technique in order to render it more suitable for industrial applications. Nanoprecipitation is still widely used to prepare particulate carriers which are based on various polymers. Biomedical applications of such drug delivery systems are multiple (Rosset et al. 2012)(Khan et al. 2012).

Table of abbreviations

Abbreviation	Definition
AmB	Amphothericin B
BBB	Blood brain barrier
CPT	Camptothecin
CUR	Curcumin
CyA	Cyclosporine A
DOX	Doxorubicin

DTX	Docetaxel
EPR	Enhanced permeability and retention
EPS	Extrapyramidal side effects
FNP	Flash nanoprecipitation
g7	Simil-opioid peptide
HA	Hyaluronic acid
HPIMM	High Pressure Interdigital Multilamination Micromixer
IOP	Intraocular pressure
LOP	Loperamide
LOP-PLGA-g7	Nanoparticles coated with simil-opioid peptide and containing loperamide
LOP-PLGA-SA-g7	Nanoparticles coated with sialic acid and simil-opioid peptide
LTZ	Letrozole
MPE	Maximal possible effect
OLZ	Olanzapine
PCL	Poly-ε-caprolactone
PDI	Polydispersity index
PEG	Poly(ethyleneglycol)
PEG-PCL	Polycaprolactone-poly(ethyleneglycol)
PES	Polyethylsebacate
PES-DOX	Polyethylene sebacate nanoparticles loaded with doxorubicin
PLA	Polylactide
PLGA	Polylactide-co-glycolide
PLGA-PEG	Polylactide-poly(ethyleneglycol)
PTX	Paclitaxel

PUL	Pullulan
PUL-PES-DOX	Polyethylene sebacate nanoparticles loaded with doxorubicin
PVA	Polyvinylalcohol
RA	All trans retinoic acid
RGD	Tripeptide arginine-glycine-aspartic acid
RGDp	Tripeptide arginine-glycine-aspartic acid peptidomimetic
RIS	Risperidone
RIV	Rivastigmine tartrate
SA	Sialic acid
SEM	Scanning electron microscope
THF	Tetrahydrofuran

1. Introduction:

Nanoprecipitation s also called solvent displacement or interfacial deposition. It is considered as one of the first developed techniques used for the encapsulation of drug molecules. This technique was developed by Fessi *et al*. (Fessi et al. 1989). Since its development, the technique has been widely used for the encapsulation of mainly, hydrophobic drugs in either nanocapsules or nanospheres. Many polymers were used for this purpose, especially, biodegragradable polyesters such as, polylactide (PLA), polylactide-co-glycolide (PLGA) and poly-ε-caprolactone (PCL). Nanocapsules are vesicular forms that exhibit core-shell structure in which the drug is mainly confined to a reservoir or within a cavity surrounded by a polymeric membrane. Nanospheres are, however, matrix-type colloidal particles in which the drug is dissolved or dispersed within the polymer matrix. The drug molecule could be also adsorbed on the surface of the nanocarrier (Mora-Huertas et al. 2010)(Letchford et Burt 2007). Nanoprecipitation is based on the interfacial deposition of polymers following the displacement of a semi-polar solvent miscible with water from a lipophilic solution (Fessi et al. 1989). It is an easy and reproducible technique that has been widely used in the preparation of nanoparticles. Nanoprecipitation has many advantages over other encapsulation techniques: (1) Simplicity (2) ease of scalability (3) good reproducibility (4) large amounts of toxic

solvents are avoided (5) obtaining of submicron particle sizes with narrow size distribution and (6) no need for using of high energy input (Lassalle et Ferreira 2007). In 2005, Bilati *et al.* developed a modified nanoprecipitation method designed for the encapsulation of hydrophilic molecules (Bilati et al. 2005). Figure 1A and Figure 1B show scanning electron microscopy images of nanoparticles prepared by nanoprecipitation.

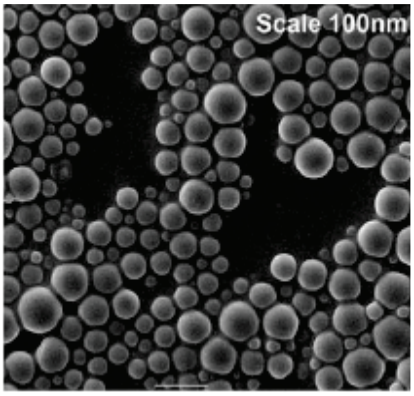


Fig. 1A. Scanning electron microscope (SEM) images of PLGA-PEG nanoparticles (Anand et al. 2010)

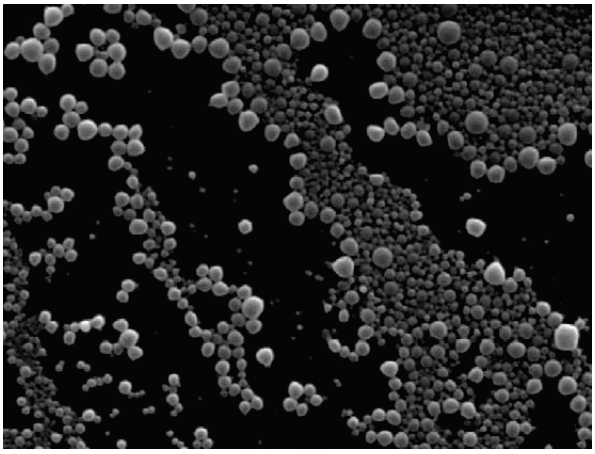


Fig. 1B. SEM image of Nanoparticles prepared by nanoprecipitation (Costantino et al. 2005)

2. Technical aspects:

2.1.Mechanism of particle formation by nanoprecipitation:

Nanoprecipitation is a simple and reproducible technique that produces particles with narrow size distribution over a wide range of processing parameters (Budhian et al. 2007). It requires two miscible phases: an organic/oil phase and an aqueous phase (See Figure 1). Lince *et al.* showed that the process of particle formation in the nanoprecipitation method includes three phases: nucleation, growth and aggregation (Lince et al. 2008). Supersaturation was described as the driving force of all these phenomena. It is defined by the ratio of polymer concentration

to polymer solubility in the organic solvent. Supersaturation is crucial because it also determines the nucleation rate. Here, fluid dynamics and mixing of phases play an important role. In fact, they influence supersaturation and owing to the rapidity of particle formation process, they determine also the nucleation rate. Consequently, poor mixing produce few big nanoparticles (low nucleation rate) while good mixing conditions give birth to high nucleation rates *i.e.* larger population of smaller nanoparticles (Lince et al. 2008). Quintanar-Guerrero *et al.*, however, explained nanoparticles formation as a result of differences in surface tension (Quintanar-Guerrero et al. 1998). This finding was based on research carried out by Davies on mass transfer between two liquids and on the Gibbs-Marangoni effect (McManamey et al. 1973)(Davies 1975). In fact, a liquid with a high surface tension (aqueous phase) pulls more strongly on the surrounding liquid than one with a low surface tension (organic phase solvent). This difference between surface tensions of the aqueous and the oil phase causes interfacial turbulence and thermal inequalities in the system. This leads to the continuous formation of vortices of solvent at the interface of both liquids. The organic solvent diffuses from regions of low surface tension which causes gradual precipitation of the polymer on the oil surface and forms nanocapsules (Mora-Huertas et al. 2010).

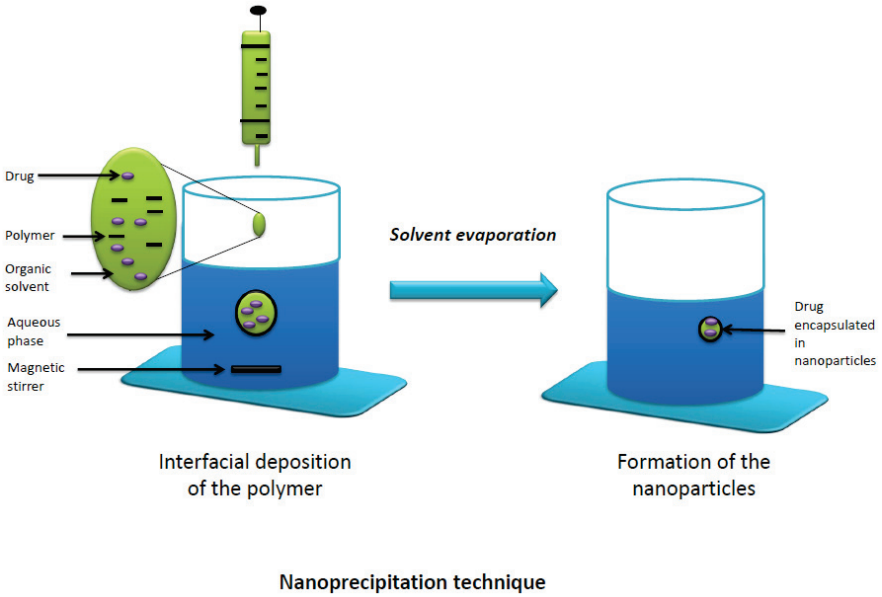


Fig. 2. The nanoprecipitation technique

2.2.Drugs :

Nanoprecipitation technique is essentially used to encapsulate hydrophobic molecules. However, some good results were also obtained with hydrophilic molecules. Table 1 contains some examples of drugs encapsulated by nanoprecipitation and their corresponding nature.

More examples will be given in the chapter by Zandanel and Charrueau (Chapter XXX). Most of the drug encapsulation studies focused either on poorly water-soluble or amphiphilic compounds that are highly soluble in water miscible organic solvents. However many studies used other approaches to allow the encapsulation of hydrophilic molecules. Three main approaches have been investigated: (1) The dissolving of the hydrophilic molecule in the external aqueous phase, (2) the use of a cosolvent or (3) the dissolution of small amounts of the molecule in the organic phase. Bilensoy *et al.* encapsulated mitomycin C in PCL based nanoparticles coated with chitosan by dissolving the hydrophilic drug in the aqueous phase (Bilensoy et al. 2009). Peltonen *et al.* used ethanol and methanol as cosolvents and added them to an aqueous solution of cromoglucate to allow drug dissolution in the organic phase (Peltonen et al. 2004). Govender *et al.* used nanoprecipitation to prepare PLGA nanoparticles containing the water soluble molecule, procaine hydrochloride. Experimental procedure consisted on the dissolution of PLGA and a specified quantity of the drug in acetonitrile (Govender et al. 1999).

Table 1. Examples of drugs encapsulated in polymer nanoparticles by nanoprecipitation

Hydrophilic molecules	***References***		***Hydrophobic molecules***	***References***
Cromoglucate	(Peltonen et al. 2004)		Olanzapine	(Seju et al. 2011)
Doxorubicin	(Sanson et al. 2010)(Han et al. 2013)		Paclitaxel	(Wang et al. 2013)
Bovine Serum Albumin	(Gao et al. 2006)		Amphotericin B	(Van de Ven et al. 2012)
Levofloxacin	(Cheow et Hadinoto 2010)		Aceclofenac	(Katara et Majumdar 2013)
10-Hydroxycamptothecin	(Zhang et al. 2007)		Curcumin	(Mazzarino et al. 2012)
Mitomycin C	(Bilensoy et al. 2009)		Retinoic acid	(Almouazen et al. 2012)
Heparin	(Eidi et al. 2010)		Naringenin	(Krishnakumar et al. 2011)
Stevioside	(Barwal et al. 2013)		Efavirenz	(Seremeta et al. 2013)
Salbutamol	(Hyvönen et al. 2005)		Naproxen	(Rosset et al. 2012)
Procaine	(Govender et al. 1999)		Chloroaluminum phthalocyanine	(Siqueira-Moura et al. 2013)

2.3.Oil phase:

The oil phase consists on an organic solvent which is miscible to water such as, ethanol or acetone. The organic phase contains also the polymer and the hydrophobic drug. Other compounds could be added to the solvent such as triglycerides, mineral or vegetable oils or hydrophobic surfactants. Addition of mineral or vegetable oils allow the obtaining of nanocapsules rather than nanospheres. Surfactants hamper the aggregation of the particulate carriers. Table 2 shows some examples of oil phases that could be used in nanoprecipitation. One can notice that acetone is the most commonly used organic solvent in nanoprecipitation.

Table 2. Examples of organic phases used in nanoprecipitation

Composition of the oil phase	**References**
Oil phases comprising one solvent	
Acetone	(Bazylińska et al. 2013) (Bernabeu et al. 2013) (Shah et al. 2014) (Siqueira-Moura et al. 2013) (Barwal et al. 2013) (Peter Christoper et al.) (Pavot et al. 2013) (Das et al. 2013a) (Cırpanlı et al. 2011) (Gupta et al. 2010) (Liu et al. 2010)(Joshi et al. 2010) (Cheng et al. 2008) (Muthu et al. 2009) (Pertuit et al. 2007) (Danhier et al. 2009a) (Çirpanli et al. 2009) (Yuan et al. 2008) (Vila et al. 2004) (Fonseca et al. 2002) (Leroueil-Le Verger et al. 1998) (Nafee et al. 2013) (Zili et al. 2005) (Yenice et al. 2008) (Memisoglu-Bilensoy et al. 2005) (Ali et al. 2013) (Zhang et Zhuo 2005) (Das et al. 2013b) (Kumar et al. 2012) (Paul et al. 2013) (Musumeci et al. 2013) (Mazzarino et al. 2012) (Eidi et al. 2012)
Ethanol	(Ubrich et al. 2005) (Perret et al. 2013a) (Perret et al. 2013b)
Ehtylacetate	(Tao et al. 2013)
Acetonitrile	(Wang et al. 2010) (Dong et Feng 2007) (Dong et Feng 2004) (Leo et al. 2004)
THF*	(de Miguel et al. 2013) (Peracchia et al. 1999) (Kaewprapan et al. 2012)
DMF*	(Suen et Chau 2013)
DMSO*	(Esfandyari-Manesh et al. 2013)
PEG*	(Ali et Lamprecht 2013)
Oil phases comprising solvent mixtures	
Acetone/ethanol	(Noronha et al. 2013) (das Neves et al. 2013) (Le Broc-Ryckewaert et al. 2013)
Acetone/methanol	(Das et Suresh 2011)
Acetone/coconut oil	(Bazylińska et al. 2013)
Solution of capric/caprylic triglyceride mixture in acetone	(Moraes et al. 2009)
Acetone and mixture of chloroform and NEt3	(Loyer et al. 2013)
Sorbitan monostearate, mineral oil and acetone	(Raffin Pohlmann et al. 2002)

THF/Water	(Kaewprapan et al. 2012)

*THF: tetrahydrofuran; DMF: Dimethylformamide; DMSO: Dimethylsulfoxide; PEG : Polyethylene Glycol.

2.4.Water phase:

The aqueous phase is usually water but some other excipients such as hydrophilic surfactants could be added to avoid particles' aggregation. These surfactants could be natural or synthetic. Likely, some polymers could be added to aqueous phase as coating materials. Hydrophilic drugs could be dissolved in the aqueous phase. Table 3 shows some examples of aqueous phases that could be used in the nanoprecipitation method. As it can be seen, the most used aqueous phase is simply water and the most used surfactant is Pluronic® F68.

Table 3. Examples of aqueous phases used in nanoprecipitation

Composition of the water phase	***References***
Water	(Esfandyari-Manesh et al. 2013) (de Miguel et al. 2013) (das Neves et al. 2013) (Suen et Chau 2013) (Das et al. 2013a) (Le Broc-Ryckewaert et al. 2013) (Le Broc-Ryckewaert et al. 2013) (Liu et al. 2010) (Danhier et al. 2009a) (Dong et Feng 2007) (Yuan et al. 2008) (Nafee et al. 2013) (Loyer et al. 2013) (Yenice et al. 2008) (Memisoglu-Bilensoy et al. 2005) (Peracchia et al. 1999) (Zhang et Zhuo 2005) (Perret et al. 2013a) (Perret et al. 2013b) (Kaewprapan et al. 2012)
Aqueous solution of Pluronic® F68	(Noronha et al. 2013) (Shah et al. 2014) (Siqueira-Moura et al. 2013) (Barwal et al. 2013) (Cırpanlı et al. 2011) (Çirpanli et al. 2009) (Dong et Feng 2004) (Leroueil-Le Verger et al. 1998) (Ubrich et al. 2005) (Das et al. 2013b) (Kumar et al. 2012) (Paul et al. 2013) (Eidi et al. 2012)
Aqueous solution of poloxamer 407	(Peter Christoper et al.) (Muthu et al. 2009)
Aqueous PVA* solution	(Ali et Lamprecht 2013)(Gupta et al. 2010) (Das et Suresh 2011) (Pertuit et al. 2007) (Tao et al. 2013)
Aqueous solution of Tween® 80	(Moraes et al. 2009) (Zili et al. 2005)
Aqueous solution of Cremophor EL	(Bazylińska et al. 2013)
Water containing TPGS*	(Bernabeu et al. 2013)
Water/ethanol	(Pavot et al. 2013) (Cheng et al. 2008)
Solution of Pluronic® F 127 in phosphate buffer (pH 9.0)	(Joshi et al. 2010)
PBS* (0.01 M, pH 7.4)	(Letchford et al. 2009)
Ethanol	(Vila et al. 2004)
Aqueous poloxamer 188 solution	(Fonseca et al. 2002)
Aqueous sodium cholate solution	(Leo et al. 2004)
Aqueous solution of polysorbate 80	(Raffin Pohlmann et al. 2002)

Aqueous sodium taurocholate solution	(Ali et al. 2013)
Water/ethanol mixture containing Tween® 80	(Musumeci et al. 2013)
Aqueous solution of acetic acid and poloxamer 188	(Mazzarino et al. 2012)

*PVA: Polyvinyl alcohol; TPGS: alphatocopheryl polyethylene glycol 1000 succinate ; PBS: Phosphate Buffer Saline

2.5.Polymers:

Numerous polymers have been used to prepare nanoparticles by nanoprecipitation. To be suitable for *in vivo* applications, polymers must be biodegradable and biocompatible. The most used materials are biodegradable polyesters such as PLGA, PCL, PLA and Eudragit®. Coating materials could also be grafted or adsorbed to the initial polymer to confer new surface properties such as, mucoadhesion, protection from reticulo-endothelial system (stealth particles) or to tune hydrophilicity. Copolymers could also be used (Miladi et al. 2014). Table 4 contains some examples of polymers used for the preparation of nanoparticles by nanoprecipitation.

Table 4. Examples of polymers used in nanoprecipitation

Polymer	***References***
PLGA	(Bazylińska et al. 2013) (Shah et al. 2014) (Siqueira-Moura et al. 2013) (Peter Christoper et al.) (Ali et Lamprecht 2013) (Das et al. 2013a) (Le Broc-Ryckewaert et al. 2013) (Cırpanlı et al. 2011) (Gupta et al. 2010) (Wang et al. 2010) (Joshi et al. 2010) (Moraes et al. 2009) (Cheng et al. 2008) (Muthu et al. 2009) (Pertuit et al. 2007) (Danhier et al. 2009a) (Çirpanli et al. 2009) (Fonseca et al. 2002) (Leroueil-Le Verger et al. 1998) (Ali et al. 2013) (Das et al. 2013b) (Paul et al. 2013) (Tao et al. 2013) (Musumeci et al. 2013)
PCL	(Noronha et al. 2013) (das Neves et al. 2013) (Cırpanlı et al. 2011) (Çirpanli et al. 2009) (Leroueil-Le Verger et al. 1998) (Zili et al. 2005) (Yenice et al. 2008) (Raffin Pohlmann et al. 2002) (Mazzarino et al. 2012)
PLA	(Barwal et al. 2013) (Pavot et al. 2013) (Leroueil-Le Verger et al. 1998) (Leo et al. 2004) (Raffin Pohlmann et al. 2002)
Eudragit® RL	(Ali et Lamprecht 2013) (Ubrich et al. 2005)
Eudragit® RS 100	(Das et Suresh 2011)
Eudragit® RS	(Ubrich et al. 2005)
Eudragit® RS PO	(Eidi et al. 2012)
PEG-PLGA	(Ali et Lamprecht 2013) (Liu et al. 2010) (Danhier et al. 2009a) (Musumeci et al. 2013)
PEG-b-PCL	(Suen et Chau 2013) (Danhier et al. 2009a) (Nafee et al. 2013)
PEG-PCL-PEG	(Zhang et Zhuo 2005)
PLA-PEG	(Vila et al. 2004)
PCL conjugated to 5-	(Pertuit et al. 2007)

aminosalicylic acid	
PCL-TPGS	(Bernabeu et al. 2013)
mPEG-PLA	(Wang et al. 2010) (Dong et Feng 2007) (Dong et Feng 2004)
MePEG-b-PCL	(Letchford et al. 2009)
PLA and hydrophobically modified Chitosan	(Yuan et al. 2008)
PBLG* derivatives	(de Miguel et al. 2013)
Amphiphilic derivatives of poly(benzyl malate)	(Loyer et al. 2013)
β-CDC6*	(Memisoglu-Bilensoy et al. 2005)
β-amphiphilic cyclodextrin	(Perret et al. 2013a)
PEGylated and non PEGylated PHDCA* polymer	(Peracchia et al. 1999)
PLGA and DOTAP*	(Kumar et al. 2012)
Dextran decanoate	(Kaewprapan et al. 2012)

*PBLG: poly(γ-benzyl-l-glutamate); β-CDC6: cyclodextrin modified on the secondary face with 6C aliphatic esters; PHDCA: poly(methoxypolyethyleneglycol cyano-acrylate-co-hexadecyl cyanoacrylate); DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane.

2.6.Influence of operating conditions:

The technique is based on the addition of one phase to the other under moderate magnetic stirring (See Figure 1). The subsequently obtained suspension of nanoparticles is subjected to evaporation of the organic solvent by a rotavapor or at ambient temperature. The next step consists of the removing of the aqueous phase either by ultracentrifugation or freeze drying. The obtained nanoparticles are characterized by the measurement of size, zeta potential and by Transmission Electron Microscopy or Scanning Electron Microscopy. Many operating conditions could exert important effect on the characteristics of the obtained nanocarriers. Effects of these parameters are summarized in Table 5.

2.6.1. Amount of polymer:

Many studies evaluated the effect of the variation of polymer amount on the characteristics of the nanoparticles. Table 5 presents some examples for the effect of polymer amount on nanoparticle characteristics. As it can be seen, an increase of polymer amount generally increased particle size and encapsulation efficiency. This could be explained by an increase of the viscosity of the oil phase which gives birth to bigger particles and render drug diffusion more difficult. According to Legrand et al., polymer concentration in organic solvent should

remain below the limit between the dilute and semi dilute regime to avoid formation of aggregates (Legrand et al. 2007).

2.6.2. Molecular weight of the polymer:

Polymer molecular weight is a crucial parameter that could exert strong influence on particles' properties. Lince *et al.* evaluated the effect of PCL molecular weight on particle size. The greater was the molecular weight, the smaller was the size of the particles. An increase of polymer molecular weight led to a decrease of particles size from 144.1 nm to 93.6 nm. This phenomenon was explained by faster precipitation of the high molecular weight PCL owing to its more limited solubility in the acetone/water medium (Lince et al. 2008)(Seremeta et al. 2013). Conversely, Blouza *et al.* reported an increase of particles size following an increase of polymer molecular weight. This finding was explained by higher viscosity of the organic solution in the case of high polymer molecular weight (Limayem Blouza et al. 2006). In another study, Legrand et al. showed no influence of the molecular weight of PLA on the size of nanoparticles produced in the absence of surfactant. In contrast, they found that the yield of formation of nanoparticles was greatly influenced by the molecular weight of the polymer highlighting that there is an optimal molecular weight of PLA to obtain high production rate of nanoparticles. It was suggested that all PLA chains with molecular weight outside the optimal range are precipitating as aggregates and contribute to reduce the yield of production of nanoparticles (Legrand et al. 2007).

2.6.3. Amount of surfactant:

Stabilizer amount influence on particle properties has been largely studied. An increase in size of PLGA nanoparticles at high polyvinylalcohol (PVA) concentrations (5-10%) has been reported by (Zweers et al. 2003) and (Arica et Lamprecht 2005), while Allemann *et al.* reported a continuous decrease in particle size (Allémann et al. 1992). Lamprecht *et al.* noticed also that an increased sodium cholate concentration led to a particle size reduction (Lamprecht et al. 2001). In order to explain this contradiction, (Budhian et al. 2007) and (Arica et Lamprecht 2005) proposed the presence of two competing effects at high PVA concentrations: an enhanced interfacial stabilization that caused a size decrease and an increased viscosity of the aqueous phase which led to a less favorable mixing efficiency and thus, to a size increase. The concentration of PVA at which one effect starts dominating over the other depends on the system and processing parameters. For PLGA nanoparticles, the size

first decreased due to better stabilization and then increased at higher PVA concentrations due to high aqueous phase viscosity (Arica et Lamprecht 2005)(Budhian et al. 2007). Guhagarkar *et al.* noticed a sharp decrease in particle size from greater than 1,000 nm to around 300 nm as PVA concentration increased from 0.1% to 0.5%. Further increase in PVA concentration to 4% resulted in an increase in particle size. In fact, the subsequent increase in viscosity of external aqueous phase hampered effective diffusion of organic phase leading to larger droplet formation and thus, an increase of mean size (Guhagarkar et al. 2009). Similar results at higher PVA concentrations have been reported (Quintanar-Guerrero et al. 1996) (Moinard-Chécot et al. 2008)(Murakami et al. 1997).

Stabilizer nature is another crucial parameter that could have an impact on particle size. For instance, Van de Ven *et al.* showed that smaller nanoparticles were prepared using Poloxamer 188 in combination with sodium cholate, whereas the largest ones were obtained with PVA (Van de Ven et al. 2012). Likely, studies performed by Limayem Blouza *et al.* and Khayata *et al.* showed that surfactant type changed the size of vitamin E-loaded nanocapsules as Tween® 80 gave the smallest particles (Limayem Blouza et al. 2006)(Khayata et al. 2012a).

2.6.4. Oil to water phase ratio:

Fonseca *et al.* reported that doubling the aqueous phase volume resulted in a significant decrease in the size of PLGA nanoparticles (Fonseca et al. 2002). In fact, in nanoprecipitation, the nanoparticles are formed due to rapid solvent diffusion to the aqueous phase (Quintanar-Guerrero et al. 1997). Consequently, as the volume of the aqueous phase increases, the diffusion of the organic solvent in the aqueous phase increases which decreases particle size. Additionally, an increase of the aqueous phase volume increases the drug amount that can be dissolved in the aqueous phase, which causes more drug loss into the aqueous phase (Budhian et al. 2007).

2.6.5. Solvents nature and order of phases' addition:

Choice of solvents depends on requirements of the method and physico-chemical properties of the polymer. In fact, organic solvent must respond to 3 criteria: (1) dissolving capacity toward polymer (2) miscibility with water and (3) low boiling point in order to facilitate evaporation. Aqueous phase consists, however, of a non solvent for the polymer. This phase would thus cause polymer precipitation to form nanoparticles. It was shown that theta solvent (a solvent in which polymer coils act like ideal chains) tends to give smaller nanoparticles than other solvents (Flory 1969)(Legrand et al. 2007). The nature of the aqueous and oil phase

and the order of phases' addition could strongly influence nanoparticles' properties. For instance, influence of aqueous phase pH was described by Govender *et al.* who reported an increasing drug entrapment and drug content trend due to an increase of aqueous phase pH from 5.8 to 9.3. In fact, aqueous phase pH influenced the ionization of the encapsulated drug, procaine hydrochloride and hence, its solubility. Consequently, an increase of the aqueous phase pH decreased the solubility of procaine hydrochloride and enhanced drug entrapment into nanoparticles (Govender et al. 1999). The effect of oil nature was also evaluated by (Khayata et al. 2012a) who noticed that nanoparticles prepared with castor oil were the largest ones. This was explained by the higher viscosity of this oil. In fact, it was shown that as oil viscosity was higher, dispersed phase viscosity increased. Polydispersity index (PDI) also augmented when the oil viscosity increased. This finding was similar to results reported by Raffin Pohlmann *et al.* who noticed an increase in particle diameter and PDI with an increase of oil viscosity (Raffin Pohlmann et al. 2002)(Khayata et al. 2012a). Effect of organic solvent nature was evaluated by other studies that had shown that solvents of high polarity like acetone gave birth to small nanoparticles by promoting rapid diffusion to the aqueous phase (Legrand et al. 2007)(Thioune et al. 1997). It was shown that a lower dielectric constant of the organic solvent resulted in larger particles size (Bilati et al. 2005). Guhagarkar *et al.* compared particles size and entrapment efficiency of polyethylene sebacate (PES) based nanoparticles. Particle size decreased significantly when tetrahydrofuran (THF) and acetone were used in combination as solvent compared to THF alone at all polymer concentrations. This was explained by more rapid diffusion of the more polar solvent acetone into the nonsolvent phase that favored the formation of smaller nanoparticles. In fact, the dielectric constant of THF/acetone (1:1) was found to be 14.5 compared to 7.5 for THF alone. In addition, increased diffusivity of the organic solvent due to addition of acetone could cause leaching of the drug into the aqueous phase thus, decreasing encapsulation efficiency (Guhagarkar et al. 2009). The order of phases' addition seems also to exert an effect on particles characteristics. The effect of adding the aqueous phase into the organic phase versus adding the organic phase into the aqueous phase was determined by Khayata *et al* who prepared vitamin E-loaded nanocapsules. Obvious aggregation between particles was observed when the aqueous phase was added to the organic phase. This was explained by the presence of the stabilizer in the aqueous phase that plays an important role in stabilizing the nanocapsule formed. This aggregation disappeared when organic phase was added to the aqueous phase (Khayata et al. 2012a). Bilati et al. used proposed a nanoprecipitaion technique which is intended to hydrophilic drugs encapsulation. Used solvents consisted of polar aprotic

solvents, ketones or esters. Dimethylsulfoxide was described as interesting solvent especially for protein dissolution. Non-solvent was chosen on the basis of its polarity in order to enhance final drug loading. Here, alcohols were shown to be suitable non solvents that could provide nanoparticles with different sizes. The same mechanism described previously for the particles formation is involved in particles formation as miscible solvents are always used (Bilati et al. 2005).

2.6.6. Stirring rate:

In nanoprecipitation, the most common used stirring method is magnetic stirring. An increase of the stirring rate generally results in a decrease in the particles' size. This is explained by more efficient shear mixing and thus, more rapid diffusion of the organic solvent to the water phase (Asadi et al. 2011).

One can conclude that many operating parameters have to be managed to obtain nanoparticles bearing good characteristics. Table 6 contains some approaches to be followed to monitor major particles properties.

Table 5. Influence of operating conditions on nanoparticles' properties

Operational parameter	**Action**	**Effect**	**References**
Drug amount	Increase	No significant effect on particles size	(Chorny et al. 2002)
		Increase of particles size	(Govender et al. 1999)(Khayata et al. 2012a)
		No significant effect on drug loading	(Chorny et al. 2002)
Polymer amount	Increase	Increase of particles size	(Chorny et al. 2002)(Limayem Blouza et al. 2006)(Simşek et al. 2013)(Dong et Feng 2004)(Ali et al. 2013)(Bazylińska et al. 2013)(Khayata et al. 2012a)(Lince et al. 2008)(Plasari et al. 1997)(Nehilla et al. 2008)(Guhagarkar et al. 2009)
		Increase of drug loading	(Chorny et al. 2002)(Dong et Feng 2004)
Polymer molecular	Increase	Increase of particles size	(Limayem Blouza et al.

weight**			2006)(Holgado et al. 2012)
		Decrease of particles size	(Seremeta et al. 2013)
		No significant effect on particles size	(Budhian et al. 2007)
		No significant effect on drug loading	(Budhian et al. 2007)
Oil to water phase ratio	Decrease	Decrease of particles size	(Budhian et al. 2007)(Bazylińska et al. 2013)(Fonseca et al. 2002)
		Increase of particles size	(Limayem Blouza et al. 2006)(Nehilla et al. 2008)
		No significant effect on particles size	(Chorny et al. 2002)
	Increase	Decrease of drug loading	(Budhian et al. 2007)(Limayem Blouza et al. 2006)(Guhagarkar et al. 2009)
		Decrease of particles size	(Dong et Feng 2004)
		Increase of particles size	(Stainmesse et al. 1995)
Organic phase addition rate	Increase	Decrease of the particles size	(Lince et al. 2008)
Surfactant amount	Increase	Decrease of the particles size	(Contado et al. 2013)(Siqueira-Moura et al. 2013)(Guhagarkar et al. 2009)
		Decrease then increase in particles size	(Budhian et al. 2007) (Limayem Blouza et al. 2006)(Khayata et al. 2012a)
		No significant effect on Particles size	(Dong et Feng 2004)
		No significant effect on drug loading	(Budhian et al. 2007)
Stirring rate	Increase	Decrease	(Asadi et al. 2011)
Organic solvent evaporation rate	Increase	No significant effect on particles size No significant effect on drug loading	(Chorny et al. 2002) (Chorny et al. 2002)

* *Yield of nanoparticle formation increases while concentration of polymer remains in the dilute regime* (Legrand et al. 2007)

** *Yield of nanoparticle production decrease when polymer Molecular weight diverge from the optimal value.* (Legrand et al. 2007)

Table 6. Principles and parameters that control particle size and drug content for nanoparticles prepared by nanoprecipitation (from (Budhian et al. 2007) with modifications)

	Principles	***Parameters***
Decrease particle size	Increase shear stress	Increase stirring rate Increase volume of aqueous phase Decrease polymer concentration in organic phase Increase surfactant concentration in aqueous phase Decrease polymer molecular weight
Increase particle size	Increase shear stress	Decrease stirring rate Decrease volume of aqueous phase Increase polymer concentration in organic phase Decrease surfactant concentration in aqueous phase Increase polymer molecular weight
Increase drug loading	Inhibit drug diffusion during organic solvent evaporation	Increase particle size Decrease relative volume of organic solvent Increase polymer concentration in organic phase Intermediate polymer molecular weight Select organic solvent with intermediate drug-solvent interactions Reduce drug solubility in the aqueous phase (alter pH)
	Increase drug-polymer interaction	Include specific interactions between drug and polymer end groups

3. Innovative approaches using nanoprecipitation:

Since the first discovery of the technique, many efforts have been made to improve its reproducibility, scalability and safety. Enhancement of reproducibility could minimize inter-batch variations while improvement of scalability allows the obtaining of formulations which are easily applicable in the pharmaceutical industry. Safety could be provided by avoiding the use of toxic organic solvents. Most common approaches are presented in Table 7. They consisted on the use of innovative mixing devices such as, “T”-shape mixer (Briancon et al. 1999), membrane contactor (Khayata et al. 2012b), microfluidics (Bally et al. 2012) or flash nanoprecipitation technique (D’Addio et Prud’homme 2011).

3.1.Membrane emulsification:

Scalability is one of the major encountered limitations in the manufacture of nanoparticles. Conventional nanoprecipitation did not allow the production of large scale batches. Membrane contactor could be an interesting alternative in such cases. The technique is relatively simple and could be used to produce large volumes of colloidal dispersions (Yedomon et al. 2013). It has also been shown to be suitable for the preparation of polymeric nanoparticles (Charcosset et Fessi 2005)(Limayem Blouza et al. 2006)(Khayata et al. 2012b). Membrane emulsification involves the permeation of the dispersed phase through a porous membrane into a tangentially moving continuous phase (See Figures 3A and 3B). The organic phase is pressed through the membrane pores allowing the formation of small droplets. The precipitation occurs between the droplets of the organic phase and the aqueous phase flowing tangentially to the membrane surface (Khayata et al. 2012b). Khayata *et al.* performed accelerated stability studies on vitamin E-loaded nanocapsules prepared by conventional nanoprecipitaion and by a membrane contactor. These studies showed good physical and chemical stability for both particles. However, nanocapsules prepared by conventional nanoprecipitation were stable for a longer time (Khayata et al. 2012b).

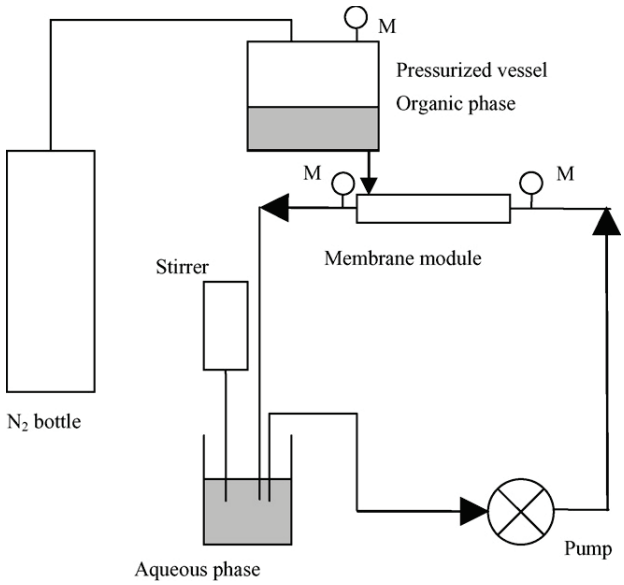


Fig.3A. Experimental set up of the membrane contactor technique (Limayem Blouza et al. 2006)

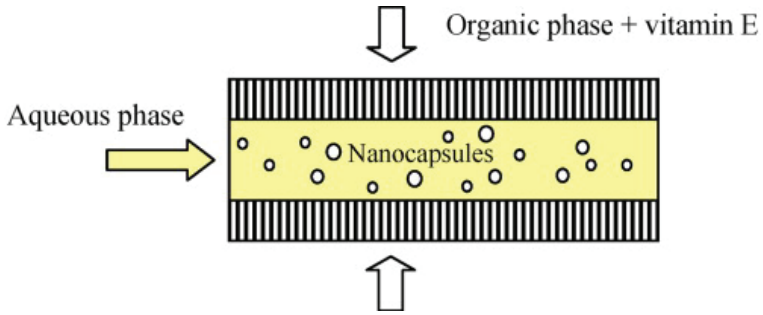


Fig. 3B. The membrane module (Khayata et al. 2012b)

Table 7. Applications of innovative approaches to obtain nanoparticles based on nanoprecipitation carried out with a mixing device.

Technique	***Drug***	***Polymer***	***Oil phase***	***Water phase***	***Size (nm)***	***Zeta potential (mV)***	***References***
"T" shape mixer	-	Eudragit®	Acetone/iso propanol mixture	Aqueous solution of surfactant	100-500	-	(Briancon et al. 1999)
Membrane contactor	Vitamin E	PCL	Acetone	Aqueous solution of Tween80	250-353	-20-(-15)	(Khayata et al. 2012b)
Membrane contactor	Vitamin E	PCL	Acetone	Aqueous solution of Tween80	170-393	-19.4-(-12.4)	(Khayata et al. 2012a)
Microfluidics	-	Linear polymers are poly(methyl methacrylate)s and branched polymers	THF containing a non-ionic surfactant (Cremophor ELP®)	Water	76-217	-	(Bally et al. 2012)
Flash nanoprecipitation	β-caroten e	Polystyrene-block-poly(ethylen e oxide)	THF	Water	80-1000	-	(Johnson et Prud'hom me 2003a)
Flash nanoprecipitation	-	PMMAs* with coumarin side functionality (PCM)	THF	Water	140-320	-	(Chung et al. 2013)
Flash nanoprecipitation	-	poly(MePE GCA-co-HDCA))*	Acetone	Water	100-300	-50-(-8)	(Valente et al. 2012)

* PMMAs : polymethyl-methacrylic acids ; poly(MePEGCA-co-HDCA)): poly(methoxy polyethylene glycol cyanoacrylate-co-hexadecyl-cyanoacrylate)

3.2.Microfluidics device:

Nanoprecipitation is usually performed via one-pot pouring of the polymer solution into the non-solvent, or by dropwise addition of one phase into the other. Microfluidic processes, using a hydrodynamic flow-focusing set-up (Karnik et al. 2008)(Rhee et al. 2011) or a confined impinging jet reactor (Johnson et Prud'homme 2003b)(Lince et al. 2011)(Nagasawa et al. 2005) have emerged to improve the mixing of the two phases. Bally *et al.* used a continuous-flow nanoprecipitation process in which, a diluted polymer solution and water were separately pumped and nanoprecipitation occurred within the micromixer. The latter consisted either of either a T-junction or a High Pressure Interdigital Multilamination Micromixer (HPIMM) (See Figure 4 for HPIMM). The obtained suspension of nanoparticles could be collected at the outlet of the micromixer (Bally et al. 2012).

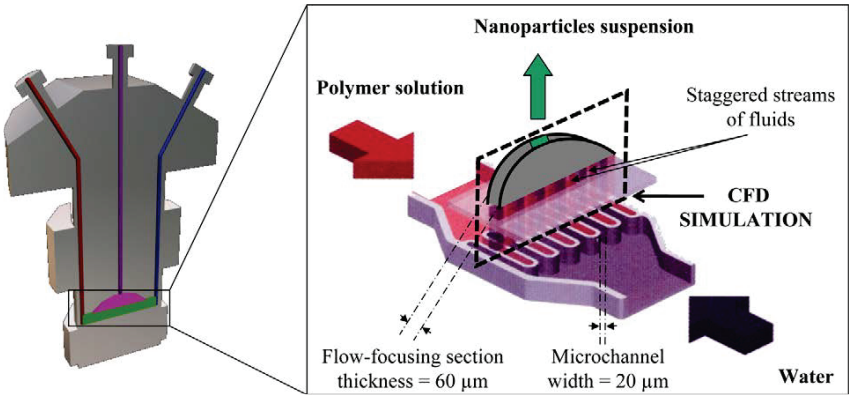


Fig. 4. Overview of HPIMM inner microstructure, used for nanoprecipitation (Bally et al. 2012).

Effect of the proportion of solvent and non-solvent which is defined by the parameter R was investigated by Bally *et al.*

$$R = \frac{Volume\ folw\ rate\ (water)}{Volume\ flow\ rate\ (polymer\ solution)}$$

It was shown that R managed the number of formed particles whatever was the mechanism considered. In nucleation mechanism, increasing R leads to higher supersaturation and more nuclei, which decreases the final particle size. In the 'mechanical' mechanism, a higher value of R increases the potential interface and more droplets are formed during phase separation. As a consequence, the local concentration of the polymer is decreased which leads to smaller nanoparticles. It was shown also that particles size depended both on initial polymer concentration (C) and on the value of R. At low R value, (R=3), particle size did not

significantly change at variable C. This was explained by the presence of two competing mechanisms which are nucleation and growth mechanism. Nucleation rate was shown to increase with C which decreased particle size. Conversely, at high polymer concentrations (≥1 wt%), growth phenomena appeared due to proximity of polymer chains. It was concluded that higher nucleation rate finally compensated with higher growth probability when C increases. However, following an increase of R to 10, size of the particles increased from 106 to 210 nm with C. This significant difference was attributed to more aggregation at high polymer concentration. Aggregation of growing particles also contributed to the increase of particle size. The effect of the mixing process on the particles size was also studied as it was previously shown to affect nanoparticles' properties (Lince et al. 2008). Bally *et al.* compared conventional T-junction, (operating via bilamination mixing) with a multilamination micromixer. Obtained data showed that bilamination mixing gave bigger particles with sizes close to ones obtained by conventional nanoprecipitation. This proves a poor mixing ability. Consequently, fine mixing was described as crucial to produce small nanoparticles at an initial polymer concentration of 1 wt%. Additionally, it was shown that micromixer-assisted nanoprecipitation gave small nanoparticles by using less non-solvent. According to Bally *et al.,* a value of R = 2 led to nanoparticles lower than 200 nm whereas at least R = 10 is required for conventional nanoprecipitation to obtain the same size. In addition, micromixing allow nanoprecipitation of polymer solution with concentrations up to 5 wt% which is impossible in conventional method in which polydisperse samples were obtained (Bally et al. 2012).

3.3.Flash nanoprecipitation (FNP):

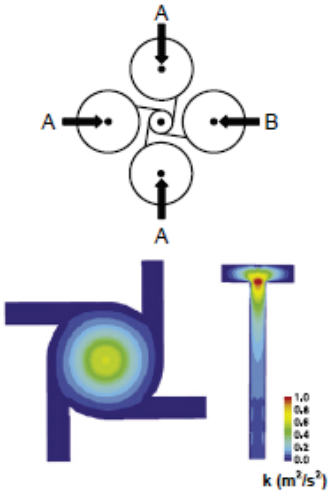


Fig. 5. A schematic representation of mutli-inlet vortex mixer used in FNP (D'Addio et Prud'homme 2011)

Simple nanoprecipitation carried out with a conventional process results in heterogeneous mixing resulting in polydispersed particle sizes. FNP, however, is a scalable process that could be used to prepare nanoparticles with controlled size distribution and a high drug loading rate. This technique was first described by Johnson and Prud'homme (Johnson et Prud'homme 2003a) to produce nanoparticles encapsulating hydrophobic drugs. FNP produces nanoparticles with a narrow size distribution ranging from 80 to 1µm. The nanoparticles are obtained via a rapid precipitation process. FNP offers also high loading capacity and the ability to encapsulate multiple drugs in the same nanoparticle. Several successful applications of FNP have been reported for encapsulation of various hydrophobic drugs, peptides, imaging agents, or a combination of both therapeutics and inorganic colloids (Chen et al. 2009)(Budijono et al.)(Kumar et al. 2010)(Shi et al. 2012). More information about the potential of this technique is given in chapter XXXX.

4. *In vivo* applications of nanoparticles designed by nanoprecipitation:

Nanoparticles designed by the nanoprecipitation technique were intended to various *in vivo* applications. Some of these formulations are summarized in Table 8, which also contains some technical aspects of the formulations such as the used polymers, the different phases and the corresponding *in vivo* application. Only recent formulations that have been assessed *in vivo* were taken into account.

4.1.Example of nanoparticles developed for cancer therapy:

Many anticancer agents were encapsulated by the use of the nanoprecipitation technique. Nanoparticles may target cancer cells by passive and active way. Passive way is related to the reduced particles size which allows nanocarriers to cross through fenestrations of endothelial cells and reach tumors. Thanks to the leaky vasculature and the poor lymphatic drainage, Enhanced Permeability and Retention effect (EPR) appears, which enhances the uptake of drugs. Active targeting, however, permits the delivery of the drug to a well-defined tissue or cell by the help of a molecular recognition which occurs between a ligand grafted on the nanoparticles and a receptor exposed on the outside of target cell surface membrane.

4.1.1. Intravenous administration:

Han *et al.* formulated Doxorubicin-loaded gelatin-co-PLA-dipalmitoyl-sn-glycero-3-phosphoethanolamine nanoparticles. *In vivo* experiments showed decreased toxicity of the

drug formulated in the developed nanoparticles compared to free Doxorubicin (DOX). In addition, it was shown that developed nanoparticles bore smaller tumor volumes than free doxorubicin when administered to mice. Nanoparticles were then more efficient and less toxic than the free drug (Han et al. 2013). Another alternative was assessed to improve DOX efficacy in liver cancer by enhancing liver targeting. In spite of being a drug of choice for hepatic carcinoma treatment, DOX hydrochloride presents major drawbacks such as the obtaining of low concentrations in the liver. Other limitations consist of cardiotoxicity, nephrotoxicity, myelosuppression and multiple drug resistance due to P-glycoprotein efflux. To circumvent those shortcomings, authors aimed to develop long circulating nanocarriers targeted to the liver. The objective was to target Asialoglycoprotein receptor (ASGPR) which is predominantly present in large numbers in the hepatocyte membrane. Polysaccharide including pullulan (PUL), was chosen as a ligand. In fact, pullulan was described to be internalized by hepatocytes via ASGPR mediated endocytosis. Polyethylene sebacate (PES) was used to encapsulate the drug. This polymer presents some advantages as its ease of synthesis, its good hydrolytic stability and low cost. *In vivo* biodistribution studies were performed on healthy female Sprague-Dawley rats. Three formulations were assessed: a DOX solution, PES nanoparticles loaded with doxorubicin (PES-DOX) and PES nanoparticles coated with PULand containing doxorubicin (PUL-PES-DOX). It was shown that PES-DOX and DOX provided higher concentrations of the drug molecule in the liver. Conversely, PUL-PES-DOX gave higher blood concentrations of the drug. These results were explained by a higher uptake of PUL-PES-DOX nanoparticles by Kupffer cells and by the prolonged circulation provided by pulluan. Authors explained Lower liver concentration of PES-DOX-PUL by a bypass of kuppfer cells. High blood concentration of PES–DOX–PUL were explained, however, by long circulating property and stealth effect conferred by pullulan. Moreover, PES-DOX and PUL-PES-DOX nanocarriers gave significantly lower heart concentration of DOX which could be interesting to reduce cardiac toxicity (Guhagarkar et al. 2010).

Lee *et al.* prepared nanoparticles based on hydrophobized pullulan (pullulan acetate) and containing paclitaxel (PTX). An *in vivo* study using HCT116 human colon carcinoma-bearing mice showed that nanoparticles reduced tumor growth more than free PTX. Efficient accumulation of nanoparticles in tumors was explained by EPR effect and the passive targeting function, although the nanoparticles did not have an active targeting ligand (Lee et al. 2012). Danhier *et al.* prepared PTX loaded and PEGylated PLGA-based nanoparticles. Tripeptide arginine-glycine-aspartic acid (RGD) has been shown to bind preferentially to

particular integrin $\alpha_v\beta_3$ which is highly expressed on tumor cells and neighboring endothelium. RGD pepetidomimetic (RGDp) was developed to mimic the activity RGD. Prepared nanoparticles were grafted either with RGD or RGDp in order to target tumor endothelium and thus, enhance the antitumor efficacy of PTX. Both of the ligands were grafted on PCL-PEG chains included in the nanoparticles. The used polymers were shown to be safe as drug-free nanoparticles resulted in the same tumor growth profile as Phosphate Buffer Saline solution. *In vivo* targeting of tumor endothelium was assessed by fluorescence studies. It was shown that fluorescence obtained following the administration RGD conjugated nanoparticles was higher than the fluorescence obtained with RGDp conjugated nanoparticles and non-conjugated nanoparticles. RGDp was, however, higher than in non conjugated nanoparticles. Furthermore, *in vivo* antitumor efficacy was evaluated in transplanted liver tumor bearing mice. Obtained data showed that RGD conjugated nanoparticles were more efficient to inhibit tumor growth than RGDp conjugated nanoparticles and non-conjugated nanoparticles. In addition, survival rate provided by RGD conjugated nanoparticles was significantly higher than RGDp conjugated nanoparticles and non targeted nanocarriers (Danhier et al. 2009b).

Docetaxel (DTX), which is a taxane, possesses an anticancer activity. This drug may cause several side effects due to its non specific action. Bone marrow depression, hypersensitivity reactions and febrile neutropenia are among those toxicological manifestations. PEGylation of carriers has emerged as a smart alternative to prolong circulation time of nanoparticles which facilitates their accumulation in tumors. In fact, stealth surface hampers binding to serum proteins and thus, recognition by reticuloendothelial system. Polycaprolactone-Polyethylene glycol (PEG-PCL) has the advantage of being approved by the Federal Drug Administration to be used clinically. Efficiency of nanoparticles was assessed in H22 tumor bearing mice (a model of hepatic cancer) and compared to the commercialized formulation of DTX Taxotere® and DTX solution. Obtained results indicated that nanocarriers significantly reduced tumor growth compared to the other formulations. In addition to enhanced uptake by cancer cells and prolonged circulating time, it was shown by *in vivo* near infrared fluorescence imaging that nanocarriers were also eliminated from other normal cells which diminished their toxicity. Penetration studies showed a passive penetration of the nanoparticles through leaky vessels surrounding cancer cells thanks to their submicron size (Liu et al. 2012).

Letrozole (LTZ) is an oral non-steroidal aromatase inhibitor indicated for the treatment of breast cancer. Mondal *et al.* prepared PLGA nanoparticles and evaluated them *in vivo* to see if nanocarriers would provide better tumor targeting. *In vivo* studies were conducted in normal

mice and Ehrlich Ascites tumor bearing mice by injection in tail vein. The blood concentration of drug-loaded nanocarriers at 24 h post-injection was three fold higher than that of free LTZ. This was explained by a solwer blood clearance of the nanoparticles. The tumor uptake of the nanoparticles was significantly higher than the free drug (1.99% of initial dose /g compared to 0.43% of initial dose/g) (Mondal et al. 2010).

4.1.2. Local administration:

Another anticancer agent, all trans retinoic acid (RA), was encapsulated in PLA based nanocapsules prepared by nanoprecipitation. Retinoic acid is an active derivative of vitamin A which can inhibit the macrophage production of inflammatory cytokines and can, thus, be indicated for some tumors where macrophages play a major role. However, RA possesses some drawbacks such as poor water solubility and low stability. It was found that nanoparticles injected intratumorally were efficiently phagocytized by glioma infiltrating macrophages (Almouazen et al. 2012). Camptothecin (CPT) is also an efficient anticancer agent. This drug presents, however, some drawbacks such as its extremely high insolubility in water and its chemical instability even in physiological pH which may lead to a loss of the pharmacological activity and cause toxic effects. Cirpanli *et al.* aimed to develop beta-cyclodextrin nanoparticles and polymeric nanoparticles (PLGA and PCL) loaded with CPT for brain cancer treatment. Antitumor efficacy of nanoparticles was assessed on a 9L rat brain tumor model. Cyclodextrin nanoparticles gave the best results (33 and 27 days as median survival time compared to 23.5 and 25.5 days for PLGA and PCL nanoparticles). This significant improvement of survival was explained by the high loading efficiency exhibited by these nanocarriers compared to other formulations (Cırpanlı et al. 2011).

4.2.Example of nanoparticles developed for Brain delivery:

Brain delivery could be alternative to treat central nervous system disorders but passage could be poor because of the presence of the Blood Brain Barrier (BBB). Many nanocarriers have been prepared to circumvent this concern and improve brain targeting. Olanzapine (OLZ), for example, is a second generation antipsychotic which is effective on the associated negative symptoms of schizophrenia. The drug, has, however, low bioavailability due to an important hepatic first-pass metabolism. In addition, OLZ presents low penetration through BBB because of an efflux by P-glycoproteins. Moreover, many side effects may appear such as hypotension, dry mouth, tremor, akathisia and somnolence. Seju *et al.* assessed nose to brain

drug delivery. *In vivo* efficiency of the prepared PLGA nanoparticles was evaluated versus a drug solution. It was shown that after 3 hours of nasal administration, nanoparticles provided a 10-fold much higher accumulation of OLZ in the brain compared to the solution form. PLGA nanocarriers showed also no significant toxicity on nasal mucosa, indicating their suitability as carriers for nasal delivery of drugs (Seju et al. 2011). Joshi *et al.* prepared PLGA nanoparticles loaded with rivastigmine tartrate (RIV) and indicated for the management of Alzheimer disease. Clinical use of RIV has shown a poor entry to the brain from blood circulation due to its hydrophilic nature. *In vivo* studies were performed in scopolamine-induced amnesic mice. An increase in learning and memory capacities was obtained for RIV solution as well as for the nanocarriers but this improvement was slower in the case of RIV solution. This was explained by better brain targeting provided by nanoparticles which could present an interesting alternative for better management of Alzheimer disease (Joshi et al. 2010). Loperamide (LOP), an opioid drug, is known to cross BBB but also to be immediately pumped back out due to the action of the P-gp. The possibility to cross the BBB and to be retained in the brain tissue may make LOP able to exert some opioid effects such as the antinociceptive activity. Tosi *et al.* prepared LOP loaded nanoparticles in order to target the brain. PLGA nanoparticles were decorated with sialic acid (SA) and/or simil-opioid peptide (g7). Two properties were then allocated to the prepared nanocarriers: First, the ability to cross the (BBB) due to the presence of g7, (a BBB-penetrating peptid) and second, the capacity to interact with SA receptors in the brain which prolongs the time of residence of the nanoparticles in the brain. This ensured a sustained pharmacological action of the encapsulated drug. *In vivo* nociceptive study was performed on male albino rats to determine the Maximal Possible Effect (MPE) to measure the intensity of the opioid effect. Two doses of nanoparticles coated with g7 (LOP-PLGA-g7) and nanocarriers coated with SA and g7 (LOP-PLGA-SA-g7) nanoparticles were assessed. It was concluded that, at both doses, nanocarriers reached rapidly the brain (15 minutes after the injection). After 30 to 60 minutes, MPE decreased then increased after 6 hours. Obtained values remained then constant for about 15 hours but diminished subsequently after 24 hours. It was shown also that pharmacological activity of LOP was prolonged compared to other formulations (Tosi et al. 2007). Moreover, LOP-PLGA-SA-g7 nanoparticles exhibited more prolonged pharmacological activity than LOP-PLGA-g7 nanoparticles. In fact, conjugation of SA modified the surface characteristics of the nanoparticles which resulted in a prolongation of the pharmacological action (Tosi et al. 2010). Risperidone (RIS) is an atypical antipsychotic agent which may cause dose-dependent extrapyramidal side effects (EPS). Consequently, the

use of low doses is necessary to avoid such manifestations. RIS is practically insoluble in water and undergoes important first-pass metabolism. Long-acting injectable formulations have been already developed but presented poor initial drug release which implied initial oral supplementation. Prepared nanoparticles were assessed *in vivo* by studying the antagonism of apomorphine-induced climbing and sniffing (antipsychotic activity) in Swiss albino mice. It was shown that PLGA nanoparticles significantly inhibited apomorphine-induced climbing and sniffing up to 72 hours while the RIS solution exhibited inhibition up to only 12 hours. Furthermore, the incorporation of the nanocarriers in an *in situ* gel system controlled the initial rapid release of RIS from nanoparticles and showed the maximum inhibition in the apomorphine-induced climbing and sniffing. This was explained by a control of the initial burst by the incorporation in the *in situ* gel. It was show also that nanoparticles significantly reduced catalepsy which is an EPS (Muthu et al. 2009).

4.3.Example of nanoparticles developed to treat Ocular diseases:

The delivery of drugs into the eye must challenge poor drug ocular bioavailability which is principally caused by precorneal loss. In fact, it was reported that barely 90% of the applied drug undergoes a pre-corneal loss by lacrimation and drainage. Precorneal loss ways include rapid tear turnover, nonproductive absorption, transient residence time in the cul-de-sac, and the relative impermeability of the drugs to the corneal epithelial membrane (Katara et Majumdar 2013). Nanoparticles have several advantages over conventional drug delivery systems intended to ocular delivery. In fact, they have slower ocular elimination and they could provide sustained release of drugs. While ocular delivery of poly(alkylcyanoacrylate) nanoparticles was described to cause disruption to the corneal epithelium cell membrane, other polymers were shown to be safe such as, PCL and Eudragit® RL. The latter has a positive charge which allows a better adhesion to eye tissue and thus, more prolonged residence time in the cornea (Das et al. 2010).

Encapsulation of melatonin in PLGA and PLGA-PEG nanoparticles was assessed for glaucoma (an optic neuropathy characterized by elevation of intraocular pressure: IOP) treatment by Musumeci *et al.* Obtained nanoparticles showed ocular tolerability in rabbit eyes. Furthermore, both formulations provided prolonged decrease in IOP but PLGA-PEG based nanopaticles were more efficient by providing greater decrease. These results were explained by the higher mucoadhesion of the PLGA-PEG nanoparticles thanks to the PEG groups. In addition, the cornea and conjunctiva have a net negative charge. Thus, the lower

negative zeta potential of PLGA-PEG nanocarriers allowed a better and more prolonged interaction with the eye (Musumeci et al. 2013). Particulate nanocarriers would be then well tolerated alternatives to prolong contact with the eye tissue. Eudragit based nanoparticles containing the anti-inflammatory drug , aceclofenac were prepared and their efficiency was evaluated *in vivo* by administration to rabbits. Eudragit® RL100 is a positively charged polymer due to many quaternary ammoniums in its structure. This property allows mucoadhesion to the anionic cornea. Katara and Majumdar assessed the effect of the prepared nanoparticles versus an aqueous solution of the drug on arachidonic acid-induced polymorphonuclear leukocytes migration and lid closure in rabbit eyes. Obtained results showed lower lid disclosure for both aceclofenac formulations but nanoparticles provided smaller lid closure compared to the drug solution. Furthermore, more enhanced anti-inflammatory effect was exerted by nanoparticles compared to drug solution (Katara et Majumdar 2013). Das *et al.* developed Eudragit® RL nanoparticles loaded with amphotericin-B (AmB) which is a polyenene antibiotic indicated in fungal keratitis. Other formulations consisting mainly of liposomes and colloidal dispersions were successfully used but presented stability concerns. Stability studies performed at room temperature and at 2-6°C showed good stability of the nanoparticles during two months. Eye irritating effects of the formulation was assessed *in vivo* in albino rabbits. All the obtained data showed that values of irritation and opaqueness were almost zero which confirmed the suitability and the safety of the formulation for ocular delivery. Positive charge of the polymer facilitated effective adhesion of the nanocarriers to the corneal surface and ensured a strong interaction with the negatively charged mucosa of the conjonctiva and the anionic mucin present in the tear film (Das et al. 2010).

Yenice *et al.* prepared hyaluronic coated PCL nanospheres containing cyclosporine A (CyA). CyA is a neutral hydrophobic peptide which is indicated for multiple ocular immune disorders. Systemic use of the drug is limited because of the various significant side effects that may appear such as, hypertension, nephrotoxicity and hepatotoxicity. Diffusion to the ocular tissue is thought to occur only when the eye is significantly inflamed. Hyaluronic acid (HA) was used due to its mucoadhesion properties which may enhance ocular residence time of cyclosporine A and thus, enhance its ocular bioavailability and prolongs its activity. *In vivo* studies were performed by topical administration of three different formulations to Male albino New Zealand rabbits: a solution of CyA in castor oil, PCL nanospheres and PCL nanospheres coated with HA. Obtained corneal concentration of CyA for nanospheres formulations were 6-8 fold higher than those of castor oil solution. HA coated nanospheres

provided significant increase in CyA corneal uptake and similar results were obtained for the conjonctival tissue (Yenice et al. 2008). Sparfloxacin is a newer-generation hydrophobic fluoroquinolone used in bacterial conjunctivitis. This drug is poorly water soluble and presents bioavailability concerns. Gupta *et al.* aimed to enhance sparfloxacin bioavailability by the preparation of PLGA naoparticles. An *in vivo* ocular retention study was performed on Male New Zealand albino rabbits. Developed nanocarriers were compared to a marketed formulation. A good spreading was observed over the entire precorneal area for both formulations but the marketed formulation showed rapid clearing from corneal region. PLGA nanoparticles, however, adhered to the cornea for a longer duration providing, thus, a more extended release of drug. Particles size seems to be the key factor to explain this prolonged residence time on the cornea as PLGA is a negatively charged polymer and is not known to be naturally mucoadhesive (Gupta et al. 2010).

4.4.Other applications:

AmB is a polyene antibiotic which is commonly indicated for invasive fungal infections and visceral leishmaniasis. This drug has a poor water solubility which limits its oral bioavailability. In addition, many side effects were described in patients receiving AmB such as fever, chills, vomiting, headache, nausea and renal malfunctions, especially with the commercialized formulation Fungizone®. Newer lipid based formulation are more tolerated but their expensiveness and the need of well-defined daily doses limited their success. Van de Ven *et al.* aimed to develop a more potent and cost-effective formulation of AmB. Hemolysis assay showed that PLGA nanoparticles were less hemolytic than drug solution and some of them were even not hemolytic at all. A selected formation was evaluated in the acute *A. fumigatus* mouse model and its potency was compared to a nanosuspension of AmB, Fungizone® and Ambiosome®. Obtained data revealed that PLGA nanoparticles reduced *A. fumigatus* more efficiently than Fungizone®. In addition, nanocarriers were about two times more efficient to clear mice organs from the fungi than Ambiosome®. The nanosuspension was, however, four times more efficient than Ambiosome®(Van de Ven et al. 2012).

The nasal route possesses many advantages over the oral and the parenteral routes in the delivery of biomacromolecules. In fact, it is non-invasive, painless, does not require sterile preparation, and allow self-administration. However, the development of drug delivery systems intended to nasal delivery must challenge poor absorption through the nasal mucosa and eventual enzymatic degradation. New generation phenylboronic acid-functionalized

glycopolymers were developed to avoid these shortcomings. Their properties are linked to the presence of boronic acid and its derivatives which could bind to glycoproteins and glycolipids within cell surfaces. Moreover, boronic acid derivatives could resist to enzymatic degradation because they exert potent inhibition toward serine proteases such as trypsin, chymotrypsin, elastase, and leucine aminopeptidase. These properties made interesting the use of these special polymers in the development of nanocarriers, especially in the case of the encapsulation of biomacromolecules. Insulin was encapsulated in poly(3-acrylamidophenylboronic acid-ran-N-maleated glucosamine) p(AAPBA-r-MAGA) copolymers and administered to mice by the intranasal route. The potency of the developed nanoparticles was compared to an insulin solution. It was concluded that the insulin solution was not able to reduce significantly glucose blood levels while a significant decrease was provided by nanoparticles. This confirmed enhanced nasal absorption of insulin provided by phenylboronic acid-functionalized glycopolymeric nanocarriers (Zhang et al. 2012). Curcumin (CUR) is a yellow pigment in the spice turmeric (*Curcuma longa*). This drug is poorly soluble in water and presents very low oral bioavailability. CUR exhibits antioxidant, anti-inflammatory, anti-survival, antiproliferative, anti-invasive and antiangiogenic activity. The assessment of the bioavailability of PLGA-PEG nanoparticles was performed in Balb/c mice versus pure CUR. Obtained results showed that serum levels of CUR provided by nanocarriers were almost two times higher than those provided by CUR solution. Moreover, nanoparticles insured a sustained release of the drug (Anand et al. 2010).

Table 8. Examples of nanoparticles prepared by the nanoprecipiation technique and assessed in vivo

Drug	**Material**	**Organic phase**	**Non organic phase**	**Size *(nm)***	**Zeta potential *(mV)***	***In vivo* application**	**Reference**
Doxorubicin	Gelatin-co-PLA-DPPE*	Acetone	Water	131.5	-	Cancer	(Han et al. 2013)
Aceclofenac	Eudragit RL100	Acetone	0.02% (w/v) Tween 80 in water	75.52-184.36	22.5-32.6	Ocular inflammation	(Katara et Majumdar 2013)
Melatonin	PLGA and PLGA-PEG	Acetone	water/ethanol mixture (1:1 v/v), containing 0.5% (w/v) of Tween 80®	-	-	Intraocular pressure	(Musumeci et al. 2013)
Retinoic acid	PLA	0.75% Miglyol in acetone	0.05% of Montanox® VG 80 in water	153.6-229.8	-10.4-(-29.4)	Glioma	(Almouazen et al. 2012)
Paclitaxel	Hydrophobized	Acetone		154.6-	-	Cancer	(Lee et al.

	pullulan	Dimethylformamide DMSO Dimethylacetamide	Water	253 nm 132.6nm 140.5 nm 127.6nm			2012)
Docetaxel	mPEG-PCL	Acetone	Water	about 70	-	Hepatocellular carcinoma	(Liu et al. 2012)
Amphotericin-B	PLGA*	DMSO*/ acetone (1:1)	Solution of a stabilizer in water	86-153	-31.4-(-9.1)	Invasive fungal infections	(Van de Ven et al. 2012)
Insulin	(p(AAPBA-r-MAGA))*	DMSO/H2O (1:2) v/v	Water	181.1-220.9	-37.8-(-17.5)	Diabetes	(Zhang et al. 2012)
Camptothecin	beta-cyclodextrin PLGA PCL	Ethanol Acetone Acetone	Water Water Water	281 187 274	-13 -0.06 -19	Cancer	(Cırpanlı et al. 2011)
Olanzapine	PLGA	Acetonitrile	0.25% (w/v) Poloxamer 407 solution in water	91.2	-23.7	Schizophrenia	(Seju et al. 2011)
Curcumin	PLGA-PEG	Acetonitrile	0.1% pluronic F-68 in water	80.9	-	Cancer	(Anand et al. 2010)
Amphotericin-B	Eudragit RL 100	Acetone/ methanol (3:1) adjusted to pH4	1% (w/v) PVA* solution in water	134.2-290	22.7-42	Fungal keratitis	(Das et al. 2010)
Doxorubicin	Polyethylene sebacate	THF*/ acetone (1:1)	Solution of 10% of Tween 80 (v/v) in water	102.8-334.5	-25-(-18)	Hepatic cancer	(Guhagarkar et al. 2010)
Sparfloxacin	PLGA	Acetone	1.5% (w/v) PVA in water	181-232	-22.8-(-22.2)	Bacterial conjunctivitis	(Gupta et al. 2010)
Rivastigmine	PLGA	Acetone	Pluronic F 127 in phosphate buffer pH 9	135.6	-23.7	Alzheimer's disease	(Joshi et al. 2010)
Letrozole	PLGA	Acetone	0.5-1% (w/v) poloxamer-188 in water	15-100	-12-(-19.5)	Breast cancer	(Mondal et al. 2010)
Loperamide	SA-GP-PLGA*	Acetone	Poloxamer 188 in water	180	-22.8	chronic neuro-diseases	(Tosi et al. 2010)
Paclitaxel	PLGA-PCL-PEG PLGA-PCL-PEG-RGD PLGA-PCL-PEG-RGDp	Acetone	Water	114 138 146	-0.36 -0.09 0.12	Ovarian and breast cancers	(Danhier et al. 2009b)
Risperidone	PLGA	Acetone	0.5% Poloxamer 407 in water	84.1-219.1	-	Psychiatric disorders	(Muthu et al. 2009)
Cyclosporin A	Hyaluronic acid adsorbed to PCL	Acetone	Water	-	-	Ocular immune disorders	(Yenice et al. 2008)

*Gelatin-co-PLA-DPPE:Gelatin-co-PLA-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; PVA: Polyvinylalcohol; THF:Tetrahydrofuran; DMSO: Dimethylsulfoxide; PLGA: Polylactide-co-glycolide; PLGA-

PEG: Pegylated polylactide-co-glycolide; SA-GP-PLGA: Sialic acid and glycopeptides conjugated PLGA; (p(AAPBA-r-MAGA)): poly(3-acrylamidophenylboronic acid-ran-N-maleated glucosamine)

Conclusion:

Several hydrophobic or hydrophilic drugs could present bioavailability, stability or unpleasant taste concerns. Encapsulation of such molecules in nanoparticles could be a very interesting alternative to solve these problems in order to enhance the efficacy of such molecules and promote patient compliance. Nanoprecipitation is a simple and reproducible technique that has been widely used for the preparation of polymeric nanoparticles intended for several biomedical applications since its first discovery. Operating conditions have to be well managed to obtain nanoparticles with suitable properties for the biomedical applications they are designed for. Several research works have been made to use nanoprecipitation in a conventional way while other works focused on the enhancement of its scalability, reproducibility and safety. Membrane technology, microfluidics and flash nanoprecipitation were introduced to achieve such purposes. Advantages of submicron carriers prepared by nanoprecipitation in the biomedical field have been confirmed *in vivo* by numerous studies. These achievements include enhanced bioavailability, better targeting and tolerance, sustained release and enhanced absorption of the drug through biological barriers.

References:

Ali H, Kalashnikova I, White MA, Sherman M, Rytting E. Preparation, characterization, and transport of dexamethasone-loaded polymeric nanoparticles across a human placental in vitro model. Int. J. Pharm. 2013;454(1):149-157.

Ali ME, Lamprecht A. Polyethylene glycol as an alternative polymer solvent for nanoparticle preparation. Int. J. Pharm. 2013;456(1):135-142.

Allémann E, Gurny R, Doelker E. Preparation of aqueous polymeric nanodispersions by a reversible salting-out process: influence of process parameters on particle size. Int. J. Pharm. 1992;87(1–3):247-253.

Almouazen E, Bourgeois S, Boussaïd A, Valot P, Malleval C, Fessi H, et al. Development of a nanoparticle-based system for the delivery of retinoic acid into macrophages. Int. J. Pharm. 2012;430(1-2):207-215.

Anand P, Nair HB, Sung B, Kunnumakkara AB, Yadav VR, Tekmal RR, et al. Design of curcumin-loaded PLGA nanoparticles formulation with enhanced cellular uptake, and increased bioactivity in vitro and superior bioavailability in vivo. Biochem. Pharmacol. 2010;79(3):330-338.

Arica B, Lamprecht A. In vitro evaluation of betamethasone-loaded nanoparticles. Drug Dev. Ind. Pharm. 2005;31(1):19-24.

Asadi H, Rostamizadeh K, Salari D, Hamidi M. Preparation of biodegradable nanoparticles of tri-block PLA-PEG-PLA copolymer and determination of factors controlling the particle size using artificial neural network. J. Microencapsul. 2011;28(5):406-416.

Bally F, Garg DK, Serra CA, Hoarau Y, Anton N, Brochon C, et al. Improved size-tunable preparation of polymeric nanoparticles by microfluidic nanoprecipitation. Polymer. 2012;53(22):5045-5051.

Barwal I, Sood A, Sharma M, Singh B, Yadav SC. Development of stevioside Pluronic-F-68 copolymer based PLA-nanoparticles as an antidiabetic nanomedicine. Colloids Surf. B Biointerfaces. 2013;101:510-516.

Bazylińska U, Lewińska A, Lamch Ł, Wilk KA. Polymeric nanocapsules and nanospheres for encapsulation and long sustained release of hydrophobic cyanine-type photosensitizer. Colloids Surf. Physicochem. Eng. Asp. [Internet]. mars 2013 [cité 1 mai 2013]; Disponible sur: https://www-sciencedirect-com.frodon.univ-paris5.fr/science/article/pii/S092777571300126X

Bernabeu E, Helguera G, Legaspi MJ, Gonzalez L, Hocht C, Taira C, et al. Paclitaxel-loaded PCL-TPGS nanoparticles: In vitro and in vivo performance compared with Abraxane(®). Colloids Surf. B Biointerfaces. 2013;113C:43-50.

Bilati U, Allémann E, Doelker E. Development of a nanoprecipitation method intended for the entrapment of hydrophilic drugs into nanoparticles. Eur. J. Pharm. Sci. 2005;24(1):67-75.

Bilensoy E, Sarisozen C, Esendağli G, Doğan AL, Aktaş Y, Sen M, et al. Intravesical cationic nanoparticles of chitosan and polycaprolactone for the delivery of Mitomycin C to bladder tumors. Int. J. Pharm. 2009;371(1-2):170-176.

Briancon S, Fessi H, Lecomte F, Lieto J. Study of an original production process of nanoparticles by precipitation. Récents Prog. En Génie Procédés [Internet]. 1999 [cité 27 août 2014]. p. 157-164. Disponible sur: http://cat.inist.fr/?aModele=afficheN&cpsidt=1142605

Le Broc-Ryckewaert D, Carpentier R, Lipka E, Daher S, Vaccher C, Betbeder D, et al. Development of innovative paclitaxel-loaded small PLGA nanoparticles: study of their antiproliferative activity and their molecular interactions on prostatic cancer cells. Int. J. Pharm. 1 oct 2013;454(2):712-719.

Budhian A, Siegel SJ, Winey KI. Haloperidol-loaded PLGA nanoparticles: systematic study of particle size and drug content. Int. J. Pharm. 24 mai 2007;336(2):367-375.

Budijono SJ, Shan J, Yao N, Miura Y, Hoye T, Austin RH, et al. Synthesis of Stable Block-Copolymer-Protected NaYF4:Yb3+, Er3+ Up-Converting Phosphor Nanoparticles. Chem. Mater. 22(2):311-318.

Campos EVR, de Melo NFS, de Paula E, Rosa AH, Fraceto LF. Screening of Conditions for the Preparation of Poly(-Caprolactone) Nanocapsules Containing the Local Anesthetic Articaine. J. Colloid Sci. Biotechnol. 2013;2(2):106-111.

Charcosset C, Fessi H. Preparation of nanoparticles with a membrane contactor. J. Membr. Sci. 2005;266(1–2):115-120.

Chen T, D'Addio SM, Kennedy MT, Swietlow A, Kevrekidis IG, Panagiotopoulos AZ, et al. Protected peptide nanoparticles: experiments and brownian dynamics simulations of the energetics of assembly. Nano Lett. 2009;9(6):2218-2222.

Cheng F-Y, Wang SP-H, Su C-H, Tsai T-L, Wu P-C, Shieh D-B, et al. Stabilizer-free poly(lactide-co-glycolide) nanoparticles for multimodal biomedical probes. Biomaterials. 2008;29(13):2104-2112.

Cheow WS, Hadinoto K. Enhancing encapsulation efficiency of highly water-soluble antibiotic in poly(lactic-co-glycolic acid) nanoparticles: Modifications of standard nanoparticle preparation methods. Colloids Surf. Physicochem. Eng. Asp. 2010;370(1-3):79-86.

Chorny M, Fishbein I, Danenberg HD, Golomb G. Lipophilic drug loaded nanospheres prepared by nanoprecipitation: effect of formulation variables on size, drug recovery and release kinetics. J. Controlled Release. 2002;83(3):389-400.

Chung JW, Neikirk C, Priestley RD. Investigation of coumarin functionality on the formation of polymeric nanoparticles. J. Colloid Interface Sci. 2013;396:16-22.

Cırpanlı Y, Allard E, Passirani C, Bilensoy E, Lemaire L, Calış S, et al. Antitumoral activity of camptothecin-loaded nanoparticles in 9L rat glioma model. Int. J. Pharm. 2011;403(1-2):201-206.

Çirpanli Y, Bilensoy E, Lale Doğan A, Çaliş S. Comparative evaluation of polymeric and amphiphilic cyclodextrin nanoparticles for effective camptothecin delivery. Eur. J. Pharm. Biopharm. 2009;73(1):82-89.

Contado C, Vighi E, Dalpiaz A, Leo E. Influence of secondary preparative parameters and aging effects on PLGA particle size distribution: a sedimentation field flow fractionation investigation. Anal. Bioanal. Chem. 2013;405(2-3):703-711.

Costantino L, Gandolfi F, Tosi G, Rivasi F, Vandelli MA, Forni F. Peptide-derivatized biodegradable nanoparticles able to cross the blood-brain barrier. J. Control. Release Off. J. Control. Release Soc. 2005;108(1):84-96.

D'Addio SM, Prud'homme RK. Controlling drug nanoparticle formation by rapid precipitation. Adv. Drug Deliv. Rev. 2011;63(6):417-426.

Danhier F, Lecouturier N, Vroman B, Jérôme C, Marchand-Brynaert J, Feron O, et al. Paclitaxel-loaded PEGylated PLGA-based nanoparticles: In vitro and in vivo evaluation. J. Controlled Release. 2009 a;133(1):11-17.

Danhier F, Vroman B, Lecouturier N, Crokart N, Pourcelle V, Freichels H, et al. Targeting of tumor endothelium by RGD-grafted PLGA-nanoparticles loaded with Paclitaxel. J. Controlled Release. 2009 b;140(2):166-173.

Das S, Das J, Samadder A, Paul A, Khuda-Bukhsh AR. Strategic formulation of apigenin-loaded PLGA nanoparticles for intracellular trafficking, DNA targeting and improved therapeutic effects in skin melanoma in vitro. Toxicol. Lett. 2013 a;223(2):124-138.

Das S, Das J, Samadder A, Paul A, Khuda-Bukhsh AR. Efficacy of PLGA-loaded apigenin nanoparticles in Benzo[a]pyrene and ultraviolet-B induced skin cancer of mice: Mitochondria mediated apoptotic signalling cascades. Food Chem. Toxicol. Int. J. Publ. Br. Ind. Biol. Res. Assoc. 2013 b;62:670-680.

Das S, Suresh PK. Nanosuspension: a new vehicle for the improvement of the delivery of drugs to the ocular surface. Application to amphotericin B. Nanomedicine Nanotechnol. Biol. Med. 2011;7(2):242-247.

Das S, Suresh PK, Desmukh R. Design of Eudragit RL 100 nanoparticles by nanoprecipitation method for ocular drug delivery. Nanomedicine Nanotechnol. Biol. Med. 2010;6(2):318-323.

Davies JT. Local eddy diffusivities related to « bursts » of fluid near solid walls. Chem. Eng. Sci. 1975;30(8):996-997.

Dong Y, Feng S-S. In vitro and in vivo evaluation of methoxy polyethylene glycol–polylactide (MPEG–PLA) nanoparticles for small-molecule drug chemotherapy. Biomaterials. 2007;28(28):4154-4160.

Dong Y, Feng S-SS-S. Methoxy poly(ethylene glycol)-poly(lactide) (MPEG-PLA) nanoparticles for controlled delivery of anticancer drugs. Biomaterials. 2004;25(14):2843-2849.

Eidi H, Joubert O, Attik G, Duval RE, Bottin MC, Hamouia A, et al. Cytotoxicity assessment of heparin nanoparticles in NR8383 macrophages. Int. J. Pharm. 30 août 2010;396(1-2):156-165.

Eidi H, Joubert O, Némos C, Grandemange S, Mograbi B, Foliguet B, et al. Drug delivery by polymeric nanoparticles induces autophagy in macrophages. Int. J. Pharm. 2012;422(1-2):495-503.

Esfandyari-Manesh M, Ghaedi Z, Asemi M, Khanavi M, Manayi A, Jamalifar H, et al. Study of antimicrobial activity of anethole and carvone loaded PLGA nanoparticles. J. Pharm. Res. 2013;7(4):290-295.

Fessi H, Puisieux F, Devissaguet JP, Ammoury N, Benita S. Nanocapsule formation by interfacial polymer deposition following solvent displacement. Int. J. Pharm. 1989 a;55(1):R1-R4.

Fessi H, Puisieux F, Devissaguet JP, Ammoury N, Benita S. Nanocapsule formation by interfacial polymer deposition following solvent displacement. Int. J. Pharm. 1989 b;55(1):R1-R4.

Flory PJ. Statistical mechanics of chain molecules. Interscience Publishers; 1969.

Fonseca C, Simões S, Gaspar R. Paclitaxel-loaded PLGA nanoparticles: preparation, physicochemical characterization and in vitro anti-tumoral activity. J. Control. Release Off. J. Control. Release Soc. 2002;83(2):273-286.

Gao H, Yang Y, Fan Y, Ma J. Conjugates of poly(dl-lactic acid) with ethylenediamino or diethylenetriamino bridged bis(β-cyclodextrin)s and their nanoparticles as protein delivery systems. J. Controlled Release. 2006;112(3):301-311.

Govender T, Stolnik S, Garnett MC, Illum L, Davis SS. PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug. J. Control. Release Off. J. Control. Release Soc. 1999;57(2):171-185.

Grando CRC, Guimarães CA, Mercuri LP, Matos J do R, Santana MHA. Preparation and Characterization of Solid Lipid Nanoparticles Loaded with Racemic Mitotane. J. Colloid Sci. Biotechnol. 2013;2(2):140-145.

Guhagarkar SA, Gaikwad RV, Samad A, Malshe VC, Devarajan PV. Polyethylene sebacate–doxorubicin nanoparticles for hepatic targeting. Int. J. Pharm. 2010;401(1–2):113-122.

Guhagarkar SA, Malshe VC, Devarajan PV. Nanoparticles of Polyethylene Sebacate: A New Biodegradable Polymer. AAPS PharmSciTech.l 2009;10(3):935-942.

Gupta H, Aqil M, Khar RK, Ali A, Bhatnagar A, Mittal G. Sparfloxacin-loaded PLGA nanoparticles for sustained ocular drug delivery. Nanomedicine Nanotechnol. Biol. Med. 2010;6(2):324-333.

Han S, Li M, Liu X, Gao H, Wu Y. Construction of amphiphilic copolymer nanoparticles based on gelatin as drug carriers for doxorubicin delivery. Colloids Surf. B Biointerfaces. 2013;102:833-841.

Holgado MA, Martin-banderas, Alvarez-fuentes, Duran-lobato, Prados J, Melguizo, et al. Cannabinoid derivate-loaded PLGA nanocarriers for oral administration: formulation, characterization, and cytotoxicity studies. Int. J. Nanomedicine. 2012;5793.

Hyvönen S, Peltonen L, Karjalainen M, Hirvonen J. Effect of nanoprecipitation on the physicochemical properties of low molecular weight poly(l-lactic acid) nanoparticles loaded with salbutamol sulphate and beclomethasone dipropionate. Int. J. Pharm. 2005;295(1-2):269-281.

Johnson BK, Prud'homme RK. Flash NanoPrecipitation of Organic Actives and Block Copolymers using a Confined Impinging Jets Mixer. Aust. J. Chem. 2003 a;56(10):1021-1024.

Johnson BK, Prud'homme RK. Chemical processing and micromixing in confined impinging jets. AIChE J. 2003 b;49(9):2264-82.

Joshi SA, Chavhan SS, Sawant KK. Rivastigmine-loaded PLGA and PBCA nanoparticles: preparation, optimization, characterization, in vitro and pharmacodynamic studies. Eur. J. Pharm. Biopharm. Off. J. Arbeitsgemeinschaft Für Pharm. Verfahrenstechnik EV. 2010;76(2):189-199.

Kaewprapan K, Inprakhon P, Marie E, Durand A. Enzymatically degradable nanoparticles of dextran esters as potential drug delivery systems. Carbohydr. Polym. 2012;88(3):875-881.

Karnik R, Gu F, Basto P, Cannizzaro C, Dean L, Kyei-Manu W, et al. Microfluidic platform for controlled synthesis of polymeric nanoparticles. Nano Lett. 2008;8(9):2906-2912.

Katara R, Majumdar DK. Eudragit RL 100-based nanoparticulate system of aceclofenac for ocular delivery. Colloids Surf. B Biointerfaces. 2013;103:455-462.

Khan MS, Vishakante GD, Bathool A. Development and Characterization of Brimonidine Tartrate Loaded Eudragit Nanosuspensions for Ocular Drug Delivery. J. Colloid Sci. Biotechnol. 2012;1(1):122-128.

Khayata N, Abdelwahed W, Chehna MF, Charcosset C, Fessi H. Preparation of vitamin E loaded nanocapsules by the nanoprecipitation method: From laboratory scale to large scale using a membrane contactor. Int. J. Pharm. 2012 a;423(2):419-427.

Khayata N, Abdelwahed W, Chehna MF, Charcosset C, Fessi H. Stability study and lyophilization of vitamin E-loaded nanocapsules prepared by membrane contactor. Int. J. Pharm. 2012 b;439(1-2):254-259.

Krishnakumar N, Sulfikkarali N, RajendraPrasad N, Karthikeyan S. Enhanced anticancer activity of naringenin-loaded nanoparticles in human cervical (HeLa) cancer cells. Biomed. Prev. Nutr. 2011;1(4):223-231.

Kumar A, Wonganan P, Sandoval MA, Li X, Zhu S, Cui Z. Microneedle-mediated transcutaneous immunization with plasmid DNA coated on cationic PLGA nanoparticles. J. Control. Release Off. J. Control. Release Soc. 2012;163(2):230-239.

Kumar V, Adamson DH, Prud'homme RK. Fluorescent polymeric nanoparticles: aggregation and phase behavior of pyrene and amphotericin B molecules in nanoparticle cores. Small Weinh. Bergstr. Ger. 20 déc 2010;6(24):2907-2914.

Lamprecht A, Ubrich N, Yamamoto H, Schäfer U, Takeuchi H, Lehr CM, et al. Design of rolipram-loaded nanoparticles: comparison of two preparation methods. J. Control. Release Off. J. Control. Release Soc. 2001;71(3):297-306.

Lassalle V, Ferreira ML. PLA nano- and microparticles for drug delivery: an overview of the methods of preparation. Macromol. Biosci. 2007;7(6):767-783.

Lee SJ, Hong G-Y, Jeong Y-I, Kang M-S, Oh J-S, Song C-E, et al. Paclitaxel-incorporated nanoparticles of hydrophobized polysaccharide and their antitumor activity. Int. J. Pharm. 2012;433(1-2):121-128.

Legrand P, Lesieur S, Bochot A, Gref R, Raatjes W, Barratt G, et al. Influence of polymer behaviour in organic solution on the production of polylactide nanoparticles by nanoprecipitation. Int. J. Pharm. 2007;344(1-2):33-43.

Leo E, Brina B, Forni F, Vandelli MA. In vitro evaluation of PLA nanoparticles containing a lipophilic drug in water-soluble or insoluble form. Int. J. Pharm. 2004;278(1):133-141.

Leroueil-Le Verger M, Fluckiger L, Kim Y-I, Hoffman M, Maincent P. Preparation and characterization of nanoparticles containing an antihypertensive agent. Eur. J. Pharm. Biopharm. 1998;46(2):137-143.

Letchford K, Burt H. A review of the formation and classification of amphiphilic block copolymer nanoparticulate structures: micelles, nanospheres, nanocapsules and polymersomes. Eur. J. Pharm. Biopharm. 2007;65(3):259-269.

Letchford K, Liggins R, Wasan KM, Burt H. In vitro human plasma distribution of nanoparticulate paclitaxel is dependent on the physicochemical properties of poly(ethylene glycol)-block-poly(caprolactone) nanoparticles. Eur. J. Pharm. Biopharm. Off. J. Arbeitsgemeinschaft Für Pharm. Verfahrenstechnik EV. 2009;71(2):196-206.

Limayem Blouza I, Charcosset C, Sfar S, Fessi H. Preparation and characterization of spironolactone-loaded nanocapsules for paediatric use. Int. J. Pharm. 2006;325(1-2):124-131.

Lince F, Marchisio DL, Barresi AA. Strategies to control the particle size distribution of poly-ε-caprolactone nanoparticles for pharmaceutical applications. J. Colloid Interface Sci. 2008;322(2):505-515.

Lince F, Marchisio DL, Barresi AA. A comparative study for nanoparticle production with passive mixers via solvent-displacement: Use of CFD models for optimization and design. Chem. Eng. Process. Process Intensif. 2011;50(4):356-368.

Lira AAM, Cordo PLA, Nogueira ECF, Almeida EDP, Junior RALC, Nunes RS, et al. Optimization of Topical All-Trans Retinoic Acid Penetration Using Poly-DL-Lactide and Poly-DL-Lactide-Co-Glycolide Microparticles. J. Colloid Sci. Biotechnol. 2013;2(2):123-129.

Liu Q, Li R, Zhu Z, Qian X, Guan W, Yu L, et al. Enhanced antitumor efficacy, biodistribution and penetration of docetaxel-loaded biodegradable nanoparticles. Int. J. Pharm. 2012;430(1-2):350-358.

Liu Y, Li K, Liu B, Feng S-S. A strategy for precision engineering of nanoparticles of biodegradable copolymers for quantitative control of targeted drug delivery. Biomaterials. 2010;31(35):9145-9155.

Loyer P, Bedhouche W, Huang ZW, Cammas-Marion S. Degradable and biocompatible nanoparticles decorated with cyclic RGD peptide for efficient drug delivery to hepatoma cells in vitro. Int. J. Pharm. 2013;454(2):727-737.

Mazzaferro S, Bouchemal K, Maksimenko A, Skanji R, Opolon P, Ponchel G. Reduced Intestinal Toxicity of Docetaxel Loaded Into Mucoadhesive Nanoparticles, in Mouse Xenograft Model. J. Colloid Sci. Biotechnol. 2012;1(2):210-217.

Mazzarino L, Travelet C, Ortega-Murillo S, Otsuka I, Pignot-Paintrand I, Lemos-Senna E, et al. Elaboration of chitosan-coated nanoparticles loaded with curcumin for mucoadhesive applications. J. Colloid Interface Sci. 2012;370(1):58-66.

McManamey WJ, Davies JT, Woollen JM, Coe JR. The influence of molecular diffusion on mass transfer between turbulent liquids. Chem. Eng. Sci. 1973;28(4):1061-1069.

De Melo NFS, Campos EVR, de Paula E, Rosa AH, Fraceto LF. Factorial Design and Characterization Studies for Articaine Hydrochloride Loaded Alginate/Chitosan Nanoparticles. J. Colloid Sci. Biotechnol. 2013;2(2):146-152.

Memisoglu-Bilensoy E, Vural I, Bochot A, Renoir JM, Duchene D, Hincal AA. Tamoxifen citrate loaded amphiphilic beta-cyclodextrin nanoparticles: in vitro characterization and cytotoxicity. J. Control. Release Off. J. Control. Release Soc. 2005;104(3):489-496.

De Miguel L, Noiray M, Surpateanu G, Iorga BI, Ponchel G. Poly(γ-benzyl-l-glutamate)-PEG-alendronate multivalent nanoparticles for bone targeting. Int. J. Pharm. 2013;460(1-2):73-82.

Miladi K, Sfar S, Fessi H, Elaissari A. Drug carriers in osteoporosis: Preparation, drug encapsulation and applications. Int. J. Pharm. 2013;445(1–2):181-195.

Moinard-Chécot D, Chevalier Y, Briançon S, Beney L, Fessi H. Mechanism of nanocapsules formation by the emulsion–diffusion process. J. Colloid Interface Sci. 2008;317(2):458-468.

Mondal N, Halder KK, Kamila MM, Debnath MC, Pal TK, Ghosal SK, et al. Preparation, characterization, and biodistribution of letrozole loaded PLGA nanoparticles in Ehrlich Ascites tumor bearing mice. Int. J. Pharm. 2010;397(1-2):194-200.

Moraes CM, de Matos AP, de Paula E, Rosa AH, Fraceto LF. Benzocaine loaded biodegradable poly-(d,l-lactide-co-glycolide) nanocapsules: factorial design and characterization. Mater. Sci. Eng. B. 2009;165(3):243-246.

Mora-Huertas CE, Fessi H, Elaissari A. Polymer-based nanocapsules for drug delivery. Int. J. Pharm. 29 2010;385(1-2):113-142.

Murakami H, Kawashima Y, Niwa T, Hino T, Takeuchi H, Kobayashi M. Influence of the degrees of hydrolyzation and polymerization of poly(vinylalcohol) on the preparation and properties of poly(dl-lactide-co-glycolide) nanoparticle. Int. J. Pharm. 1997;149(1):43-49.

Musumeci T, Bucolo C, Carbone C, Pignatello R, Drago F, Puglisi G. Polymeric nanoparticles augment the ocular hypotensive effect of melatonin in rabbits. Int. J. Pharm. 2013;440(2):135-140.

Muthu MS, Rawat MK, Mishra A, Singh S. PLGA nanoparticle formulations of risperidone: preparation and neuropharmacological evaluation. Nanomedicine Nanotechnol. Biol. Med. 2009;5(3):323-333.

Nafee N, Youssef A, El-Gowelli H, Asem H, Kandil S. Antibiotic-free nanotherapeutics: Hypericin nanoparticles thereof for improved in vitro and in vivo antimicrobial photodynamic therapy and wound healing. Int. J. Pharm. 2013;454(1):249-258.

Nagasawa H, Aoki N, Mae K. Design of a New Micromixer for Instant Mixing Based on the Collision of Micro Segments. Chem. Eng. Technol. 2005;28(3):324-30.

Nehilla B, Bergkvist M, Popat K, Desai T. Purified and surfactant-free coenzyme Q10-loaded biodegradable nanoparticles. Int. J. Pharm. 2008;348(1-2):107-114.

Das Neves J, Amiji M, Bahia MF, Sarmento B. Assessing the physical-chemical properties and stability of dapivirine-loaded polymeric nanoparticles. Int. J. Pharm. 2013;456(2):307-314.

Noronha CM, Granada AF, de Carvalho SM, Lino RC, de O.B. Maciel MV, Barreto PLM. Optimization of α-tocopherol loaded nanocapsules by the nanoprecipitation method. Ind. Crops Prod. 2013;50:896-903.

Paul A, Das S, Das J, Samadder A, Khuda-Bukhsh AR. Cytotoxicity and apoptotic signalling cascade induced by chelidonine-loaded PLGA nanoparticles in HepG2 cells in vitro and bioavailability of nano-chelidonine in mice in vivo. Toxicol. Lett. 2013;222(1):10-22.

Pavot V, Rochereau N, Primard C, Genin C, Perouzel E, Lioux T, et al. Encapsulation of Nod1 and Nod2 receptor ligands into poly(lactic acid) nanoparticles potentiates their immune properties. J. Control. Release Off. J. Control. Release Soc. 2013;167(1):60-67.

Peltonen L, Aitta J, Hyvönen S, Karjalainen M, Hirvonen J. Improved entrapment efficiency of hydrophilic drug substance during nanoprecipitation of poly(l)lactide nanoparticles. AAPS PharmSciTech. 2004;5(1):E16.

Peracchia MT, Fattal E, Desmaële D, Besnard M, Noël JP, Gomis JM, et al. Stealth PEGylated polycyanoacrylate nanoparticles for intravenous administration and splenic targeting. J. Control. Release Off. J. Control. Release Soc. 1999;60(1):121-128.

Perret F, Duffour M, Chevalier Y, Parrot-Lopez H. Design, synthesis, and in vitro evaluation of new amphiphilic cyclodextrin-based nanoparticles for the incorporation and controlled release of acyclovir. Eur. J. Pharm. Biopharm. Off. J. Arbeitsgemeinschaft Für Pharm. Verfahrenstechnik EV. 2013 a;83(1):25-32.

Perret F, Marminon C, Zeinyeh W, Nebois P, Bollacke A, Jose J, et al. Preparation and characterization of CK2 inhibitor-loaded cyclodextrin nanoparticles for drug delivery. Int. J. Pharm. 2013 b;441(1-2):491-498.

Pertuit D, Moulari B, Betz T, Nadaradjane A, Neumann D, Ismaïli L, et al. 5-amino salicylic acid bound nanoparticles for the therapy of inflammatory bowel disease. J. Control. Release Off. J. Control. Release Soc. 2007;123(3):211-218.

Peter Christoper GV, Vijaya Raghavan C, Siddharth K, Siva Selva Kumar M, Hari Prasad R. Formulation and optimization of coated PLGA – Zidovudine nanoparticles using factorial design and in vitro in vivo evaluations to determine brain targeting efficiency. Saudi Pharm. J. [Internet]. [cité 23 déc 2013]; Disponible sur: http://www.sciencedirect.com/science/article/pii/S1319016413000406

Plasari E, Grisoni PH, Villermaux J. Influence of Process Parameters on the Precipitation of Organic Nanoparticles by Drowning-Out. Chem. Eng. Res. Des. 1997;75(2):237-244.

Quintanar-Guerrero D, Allémann E, Doelker E, Fessi H. A mechanistic study of the formation of polymer nanoparticles by the emulsification-diffusion technique. Colloid Polym. Sci. 1997;275(7):640-647.

Quintanar-Guerrero D, Allémann E, Fessi H, Doelker E. Preparation techniques and mechanisms of formation of biodegradable nanoparticles from preformed polymers. Drug Dev. Ind. Pharm. 1998;24(12):1113-1128.

Quintanar-Guerrero D, Fessi H, Allémann E, Doelker E. Influence of stabilizing agents and preparative variables on the formation of poly(d,l-lactic acid) nanoparticles by an emulsification-diffusion technique. Int. J. Pharm. 1996;143(2):133-141.

Raffin Pohlmann A, Weiss V, Mertins O, Pesce da Silveira N, Stanisçuaski Guterres S. Spray-dried indomethacin-loaded polyester nanocapsules and nanospheres: development, stability evaluation and nanostructure models. Eur. J. Pharm. Sci. 2002;16(4–5):305-312.

Rhee M, Valencia PM, Rodriguez MI, Langer R, Farokhzad OC, Karnik R. Synthesis of Size-Tunable Polymeric Nanoparticles Enabled by 3D Hydrodynamic Flow Focusing in Single-Layer Microchannels. Adv. Mater. 2011;23(12):H79-H83.

Rosset V, Ahmed N, Zaanoun I, Stella B, Fessi H, Elaissari A. Elaboration of Argan Oil Nanocapsules Containing Naproxen for Cosmetic and Transdermal Local Application. J. Colloid Sci. Biotechnol. 2012;1(2):218-224.

Sanson C, Schatz C, Le Meins J-F, Soum A, Thévenot J, Garanger E, et al. A simple method to achieve high doxorubicin loading in biodegradable polymersomes. J. Control. Release Off. J. Control. Release Soc. 2010;147(3):428-435.

Seju U, Kumar A, Sawant KK. Development and evaluation of olanzapine-loaded PLGA nanoparticles for nose-to-brain delivery: In vitro and in vivo studies. Acta Biomater. 2011;7(12):4169-4176.

Seremeta KP, Chiappetta DA, Sosnik A. Poly(ε-caprolactone), Eudragit® RS 100 and poly(ε-caprolactone)/Eudragit® RS 100 blend submicron particles for the sustained release of the antiretroviral efavirenz. Colloids Surf. B Biointerfaces. 2013;102:441-449.

Shah U, Joshi G, Sawant K. Improvement in antihypertensive and antianginal effects of felodipine by enhanced absorption from PLGA nanoparticles optimized by factorial design. Mater. Sci. Eng. C. 2014;35:153-163.

Shi L, Shan J, Ju Y, Aikens P, Prud'homme RK. Nanoparticles as delivery vehicles for sunscreen agents. Colloids Surf. Physicochem. Eng. Asp. 2012;396:122-129.

Simşek S, Eroğlu H, Kurum B, Ulubayram K. Brain targeting of Atorvastatin loaded amphiphilic PLGA-b-PEG nanoparticles. J. Microencapsul. 2013;30(1):10-20.

Siqueira-Moura MP, Primo FL, Espreafico EM, Tedesco AC. Development, characterization, and photocytotoxicity assessment on human melanoma of chloroaluminum phthalocyanine nanocapsules. Mater. Sci. Eng. C. 1 2013;33(3):1744-1752.

Stainmesse S, Orecchioni A-M, Nakache E, Puisieux F, Fessi H. Formation and stabilization of a biodegradable polymeric colloidal suspension of nanoparticles. Colloid Polym. Sci. 1995;273(5):505-511.

Suen W-LL, Chau Y. Specific uptake of folate-decorated triamcinolone-encapsulating nanoparticles by retinal pigment epithelium cells enhances and prolongs antiangiogenic activity. J. Control. Release Off. J. Control. Release Soc. 2013;167(1):21-28.

Tao Y, Ning M, Dou H. A novel therapeutic system for malignant glioma: nanoformulation, pharmacokinetic, and anticancer properties of cell-nano-drug delivery. Nanomedicine Nanotechnol. Biol. Med. févr 2013;9(2):222-232.

Thioune O, Fessi H, Devissaguet JP, Puisieux F. Preparation of pseudolatex by nanoprecipitation: Influence of the solvent nature on intrinsic viscosity and interaction constant. Int. J. Pharm. 1997;146(2):233-238.

Tosi G, Costantino L, Rivasi F, Ruozi B, Leo E, Vergoni AV, et al. Targeting the central nervous system: in vivo experiments with peptide-derivatized nanoparticles loaded with Loperamide and Rhodamine-123. J. Control. Release Off. J. Control. Release Soc. 2007;122(1):1-9.

Tosi G, Vergoni AV, Ruozi B, Bondioli L, Badiali L, Rivasi F, et al. Sialic acid and glycopeptides conjugated PLGA nanoparticles for central nervous system targeting: In vivo pharmacological evidence and biodistribution. J. Control. Release Off. J. Control. Release Soc. 2010;145(1):49-57.

Ubrich N, Schmidt C, Bodmeier R, Hoffman M, Maincent P. Oral evaluation in rabbits of cyclosporin-loaded Eudragit RS or RL nanoparticles. Int. J. Pharm. 2005;288(1):169-175.

Valente I, Celasco E, Marchisio DL, Barresi AA. Nanoprecipitation in confined impinging jets mixers: Production, characterization and scale-up of pegylated nanospheres and nanocapsules for pharmaceutical use. Chem. Eng. Sci. 2012;77:217-227.

Van de Ven H, Paulussen C, Feijens PB, Matheeussen A, Rombaut P, Kayaert P, et al. PLGA nanoparticles and nanosuspensions with amphotericin B: Potent in vitro and in vivo alternatives to Fungizone and AmBisome. J. Controlled Release. 10 août 2012;161(3):795-803.

Vila A, Gill H, McCallion O, Alonso MJ. Transport of PLA-PEG particles across the nasal mucosa: effect of particle size and PEG coating density. J. Controlled Release. 2004;98(2):231-244.

Wang F, Li J, Wang C. Hydrophilic and Fluorescent Colloidal Nanorods of MWNTs as Effective Targeted Drug Carrier. J. Colloid Sci. Biotechnol. 2012;1(2):192-200.

Wang G, Yu B, Wu Y, Huang B, Yuan Y, Liu CS. Controlled preparation and antitumor efficacy of vitamin E TPGS-functionalized PLGA nanoparticles for delivery of paclitaxel. Int. J. Pharm. 2013;446(1-2):24-33.

Wang J, Feng S-S, Wang S, Chen Z-Y. Evaluation of cationic nanoparticles of biodegradable copolymers as siRNA delivery system for hepatitis B treatment. Int. J. Pharm. 15 nov 2010;400(1-2):194-200.

Yedomon B, Fessi H, Charcosset C. Preparation of Bovine Serum Albumin (BSA) nanoparticles by desolvation using a membrane contactor: A new tool for large scale production. Eur. J. Pharm. Biopharm. Off. J. Arbeitsgemeinschaft Für Pharm. Verfahrenstechnik EV. 2013;85(3):398-405.

Yenice I, Mocan MC, Palaska E, Bochot A, Bilensoy E, Vural I, et al. Hyaluronic acid coated poly-epsilon-caprolactone nanospheres deliver high concentrations of cyclosporine A into the cornea. Exp. Eye Res. 2008;87(3):162-167.

Yuan X-B, Yuan Y-B, Jiang W, Liu J, Tian E-J, Shun H-M, et al. Preparation of rapamycin-loaded chitosan/PLA nanoparticles for immunosuppression in corneal transplantation. Int. J. Pharm. 2008;349(1-2):241-248.

Zhang L, Yang M, Wang Q, Li Y, Guo R, Jiang X, et al. 10-Hydroxycamptothecin loaded nanoparticles: preparation and antitumor activity in mice. J. Control. Release Off. J. Control. Release Soc. 4 2007;119(2):153-162.

Zhang X, Wang Y, Zheng C, Li C. Phenylboronic acid-functionalized glycopolymeric nanoparticles for biomacromolecules delivery across nasal respiratory. Eur. J. Pharm. Biopharm. Off. J. Arbeitsgemeinschaft Für Pharm. Verfahrenstechnik EV. 2012;82(1):76-84.

Zhang Y, Zhuo R. Synthesis and in vitro drug release behavior of amphiphilic triblock copolymer nanoparticles based on poly (ethylene glycol) and polycaprolactone. Biomaterials. 2005;26(33):6736-6742.

Zili Z, Sfar S, Fessi H. Preparation and characterization of poly-epsilon-caprolactone nanoparticles containing griseofulvin. Int. J. Pharm. 2005;294(1-2):261-267.

Zweers MLT, Grijpma DW, Engbers GHM, Feijen J. The preparation of monodisperse biodegradable polyester nanoparticles with a controlled size. J. Biomed. Mater. Res. B Appl. Biomater. 2003;66(2):559-566.